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INTRODUCTION

The reactions of phosphorus (P) compounds in the soil environment have been the subject of much study by soil scientists throughout the world. This is due to the important role that P plays in plant nutrition, being classified as a macronutrient. Plants obtain their P for growth and development from the soil solution, primarily as orthophosphate (Pi), and it is important that the concentration of Pi in this solution does not become limiting. Application of fertilizers to keep this problem from developing and the use of high analysis polyphosphates as sources of P fertilizers has increased greatly in the past few years. Polyphosphate fertilizers such as 15-62-0(15-27-0) contain about half of their P in the form of pyrophosphate.

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) is the enzyme that catalyzes the hydrolysis of inorganic pyrophosphate (PPi) to Pi. A simple and precise assay method for the study of this enzyme in the soil environment had been undertaken previously by the author and its presence was demonstrated in all soils tested. However, information in the biochemical literature indicates that divalent cations are essential for pyrophosphatase activity. An investigation of the requirement for divalent cations by inorganic pyrophosphatase of soils is, therefore, of interest because of the importance of this enzyme in supplying Pi,

from PPi in fertilizers, to the soil solution for subsequent plant uptake.

Pi can also be replenished in the soil solution by mineralization of native or added organic P compounds in soil. The rate that mineralization occurs in soil can be an important factor in supplying P for plant growth and development. Although the mineralization of soil organic P compounds is intimately bound up with that of the mineralization of organic matter in soils as a whole, we are now able, using current methods, to look at individual reactions of the P cycle in soil. Phosphatases in the soil environment are considered to play a major role in the mineralization process of organic P by catalyzing hydrolytic cleavage of inorganic phosphate groups from organic P compounds.

Organic phosphates added to soils are attacked by phosphatases in soil and the Pi produced is then susceptible to fixation reactions. It has been found that calcareous soils allow deeper infiltration into their profile of added organic phosphates than do acid soils. However, no explanation has been provided to explain this observation. Greater infiltration of organic P compounds could potentially be a very important consideration in fields where topdressing of fertilizers must occur, as in pastures or when applied in irrigation waters. The role of phosphatases in attacking organic P compounds added to soil and the factors affecting production of Pi from such compounds deserve investigation.

The sources of phosphatases operating within a soil environment are thought to be microorganisms, whole plants and plant roots, or any other substance which is added to the soil system which contains phosphatase activity. Little information is available to show whether plants, manures, or sewage sludges may contribute to phosphatase activities in soil. Do added phosphatases become stabilized and continue to show activity for a period of time after addition to soil?

One way to answer this question is to look specifically at the role of soil components in binding enzyme protein entering the soil environment. Clay minerals are prime candidates for such a study, because they are present in all soils, have large reactive surface areas, high specific charge, and previous demonstrations have shown their ability to bind proteins. If we look at the effect of clay minerals on phosphatase activity and interpret these data using kinetic theory, we may be able to determine more accurately what type of interactions are actually occurring on a molecular level. This would give us information beyond that so often reported that "clays inhibit enzyme activity" and hopefully begin answering in what manner the clay-enzyme interactions function in causing this inhibition.

The objectives of this study were: (a) to examine the effects of metal ions on inorganic pyrophosphatase activity of soils, (b) to study the hydrolysis of organic and inorganic phosphorus compounds added to soils, (c) to study the

phosphatases in plant materials, manures, sewage sludges and soils, and (d) to study the mode of inhibition of corn root acid phosphatase and inorganic pyrophosphatase by clay minerals.

LITERATURE REVIEW

Enzyme activity in soils is a result of accumulated enzymes and enzymes of proliferating microorganisms. By definition, accumulated enzymes are regarded as those present and active in a soil environment in which no microbial proliferation takes place (Kiss et al., 1975). The various sources that contribute to the total enzymatic activity of a soil are listed by Skujins (1976) as being (1) free enzymes adsorbed or otherwise bound to soil organic and inorganic matter; (2) free enzymes released into a soil from lysed microorganisms during assay procedures, including the effects of bacteriostatic and aseptic reagents; (3) enzymes accessible in dead but not lysed cells; (4) free enzymes released into a soil from plant roots or enzymes on the surface of roots; (5) free enzymes released into a soil from other plant debris, (6) any metabolic activities of live cells and roots present in a soil; (7) any metabolic activities of soil animals present in the sample; and (8) inorganic catalytic activities contributing to the apparent enzymatic reactions. One of the important unsolved problems in soil enzymology is the distinction between enzyme activity associated with the living cell and free, extracellular enzyme activity (i.e., activity associated with accumulated enzymes). There is a need for further work to establish the separation of the activity from these two sources.

Kiss et al. (1975), in their review of soil enzymes, discussed the significance accumulated enzymes play in the important C, N, P, and S cycles in soil. They concluded that enzymes accumulated in soil play an important role in the initial phases of the decomposition of organic residues, transformations of some mineral compounds, and that they can function under conditions unfavorable for the proliferation of microorganisms. It seems evident, therefore, that accumulated enzymes contribute significantly to the biological cycles of the nutrients in the environment.

The phosphorus (P) cycle in soils plays a key role in the soil-plant environment because P is an essential nutrient for plant growth and development. The P cycle in soils is not yet clearly understood, but it is generally accepted that plants take up P primarily as inorganic phosphate (Pi).

Phosphorus in soils can be divided into two fractions, inorganic and organic. The proportion of inorganic P to organic P varies from soil to soil. Analyses of surface soils have shown values for organic P ranging from as low as 0.3% of the total to as high as 95% of the total (see Black, 1968).

The inorganic forms are those in which one or more of the hydrogen atoms of phosphoric acid have been replaced by metallic cations. Examples of commonly found inorganic phosphate forms in soils are apatite and slightly soluble to insoluble compounds of Ca, Mg, Mn, Fe, and Al. The availability of this

P to plants depends on the solubility of the P compounds in the soil solution.

Organic P compounds in soil are those in which one or more of the hydrogen atoms of phosphoric acid have been replaced by an organic moeity to form an ester linkage. The identity of all the forms of organic P in soils is still incomplete due to the complex nature of organic P compounds in soil, but current estimates are that up to 60% is inositol phosphate, 2-4% nucleotide phosphates, 1% phospholipids, traces of sugar phosphates such as glucose-1-phosphate, and the remainder of the organic P is unknown (Halstead and McKercher, 1975). Since a large proportion of P in soils is organically bound, the mineralization of the organic P to inorganic P is of major agricultural and economic importance. Soil phosphatases have been considered to play a major role in this process.

Besides the role phosphatases play in the mineralization process, is the important effect they have on degradation of polyphosphates in detergents and fertilizer P compounds added to soils. A variety of P fertilizers are available commercially (Tisdale and Nelson, 1975; Phillips and Webb, 1971) but the fertilizers in the form of polyphosphates (e.g., ammonium polyphosphates) are the most attractive because of their high analysis, desirable physical properties, and usefulness in formulating liquid fertilizers. A typical granular ammonium polyphosphate of nominal grade, 15-62-0(15-27-0)

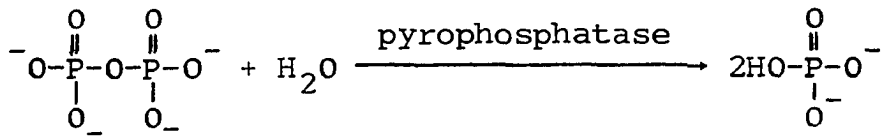
contains 41% orthophosphate, 54% pyrophosphate, 4% tripolyphosphate, and 1% tetrapolyphosphate and higher condensed phosphates (Hashimoto and Lehr, 1973). As the trend to use even higher grade fertilizers continues, condensed phosphates will supply increasing amounts of fertilizer P, and the behavior of these condensed phosphates in soil is thus of increasing interest.

The hydrolysis in soils of inorganic P compounds, such as polyphosphates, as well as organic P compounds by soil phosphatases, has been the object of much study. The general name "phosphatases" has been used to describe a broad group of enzymes that catalyze the hydrolysis of both esters and anhydrides of phosphoric acid (Schmidt and Laskowski, 1961). The Commission on Enzymes of the International Union of Biochemistry has classified all these enzymes into five major groups (Florkin and Stotz, 1964). These are the phosphoric monoester hydrolases (EC 3.1.3), phosphoric diester hydrolases (EC 3.1.4), phosphoric triester hydrolases (EC 3.1.5), enzymes acting on acid anhydride bonds (EC 3.6.1), and enzymes acting on P-N bonds (EC 3.9) such as phosphoamidases. To date, activity represented by every class has been detected in soils except for the phosphoamidases (Skujins, 1976). Much work has been done on the other phosphatases, especially the phosphoric monoester hydrolases and several excellent reviews have been written concerning these and the other phosphatases in soil (Cosgrove, 1967; Skujins, 1967, 1976; Ramirez-Martinez, 1968;

Halstead and McKercher, 1975; Kiss et al., 1975; Spier and Ross, 1978).

Inorganic Pyrophosphatase Activity of Soils

The phosphatase enzyme responsible for the hydrolysis of PPi (an important component of polyphosphate fertilizers) to Pi is pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.). The reaction can be written as follows:



Pyrophosphatases are widely distributed in nature and have been detected in yeast (Butler, 1971), bacteria, including E. coli by Josse and Wong (1971), B. megaterium by Levinson et al. (1958), Streptococcus faecalis by Oginski and Rumbaugh (1955) and Ferrobacillus ferrooxidans by Howard and Ludgren (1970), insects (Gilmour and Calaby, 1953; McElroy et al., 1951), mammalian tissues (Elliot, 1957; Naganna and Menon, 1948; Rafter, 1958), and plants (Fleury and Courtois, 1957; Naganna et al., 1955).

Pyrophosphatase activity was first detected in soil extracts by Rotini in 1933 (Kiss et al., 1975). Since then its activity in soils has been reported by Blanchar and Hossner (1969a, b), Gilliam and Sample (1968), Hashimoto and Wakefield (1974), Hughes and Hashimoto (1971), Hossner and Phillips (1971), and Douglas et al. (1976). Recently, a simple assay procedure

for the measurement of pyrophosphatase activity in soils was developed by Dick and Tabatabai (1978). They measured the amount of Pi released when incubating 1 g soil with a 50 mM PPi solution (pH 8) for 5 h at 37 C. The Pi produced was extracted with N H_2SO_4 and measured by a colorimetric procedure specific for Pi.

Previously it had been difficult to study pyrophosphatase in the soil environment because of chemical interference of PPi, it being hydrolyzed under acid conditions needed to extract the product, Pi, from soil. However, with the development of a method which could measure Pi in the presence of PPi (Dick and Tabatabai, 1977a), the parameters affecting pyrophosphatase activity in soils could be studied. A study of some of these parameters was made by Dick and Tabatabai (1978) and they found that optimal activity for soil pyrophosphatase was at pH 8. A study of kinetic properties in six Iowa soils showed that the data followed simple Michaelis-Menten kinetics and the K_m values ranged from 20 to 51 mM and the V_{max} values ranged from 130 to 826 μg Pi released · g $soil^{-1}$ · 5 h^{-1}. Inactivation temperatures for soil pyrophosphatase lie between 50 and 60 C and the energies of activation (E_a) ranged from 32.5 to 43.2 kJ · $mole^{-1}$. The influence of water-soluble Mg and Ca on pyrophosphatase in soils was studied, and it was found that the log of pyrophosphatase activity in 18 surface soils of Iowa was strongly correlated (r = 0.78**) with the water-soluble mole fraction values, Mg/(Mg + Ca) (Dick and

Tabatabai, 1979). As the water-soluble calcium levels in soil increased, the fraction Mg/(Mg + Ca) decreased, and the pyrophosphatase activity was lower. When the amount of water-soluble calcium decreased relative to magnesium, the mole fraction of Mg/(Mg + Ca) approached unity and the greatest activity occurred. Also, the amount of calcium carbonate was highly but negatively correlated with pyrophosphatase ($r = -0.97^{**}$) in five calcareous soils.

However, while searching through the literature to substantiate the finding that Mg activated and Ca inactivated pyrophosphatase, many conflicting reports were found. Searle and Hughes (1977) showed that Mg^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, and Fe^{2+} activated intracellular pyrophosphatase obtained from mixed cultures of soil microorganisms. Mg^{2+} was found to be most effective in the activation effect. These data agree with those obtained from pyrophosphatase isolated from E. coli by Josse (1966) and Ferrobacillus ferrooxidans by Howard and Lundgren (1970). They reported that there was no detectable activity in the absence of Mg^{2+} and other metal ions were poor substitutes. Activation of pyrophosphatase was not observed in the presence of Ca^{2+}, Cu^{2+}, Al^{3+}, and Fe^{3+} (Searle and Hughes, 1977). The results reported by Tono and Kornberg (1967a) and Levinson et al. (1958) for pyrophosphatase isolated from Bacillus megaterium contradict this information; they reported that activation of this enzyme occurred in the presence of Ca^{2+}.

In solutions containing both Mg^{2+} and PPi many ionic species are in equilibrium (e.g., $MgPPi^{2-}$, Mg_2PPi, PPi^{4-}). For optimal activity, the concentration of cation needed is approximately equal to that of the PPi concentration (Josse, 1966). This suggests that $MgPPi^{2-}$ is the actual substrate for pyrophosphatase, and results reported by Josse (1966) and Unemoto et al. (1973) support this hypothesis. Free PPi (not combined with metal) is not a substrate, although it is bound to the enzyme 50 times more strongly than $MgPPi^{2-}$ (Butler, 1971). Results reported by Dick and Tabatabai (1978) showed that when high levels of PPi substrate (free PPi) were added to the soil pyrophosphatase assay system, activity decreased.

Behavior of Soluble Organic Phosphates in Soils

The use of inorganic phosphate compounds as fertilizer sources of P for plant growth and development is a common agricultural practice. The role that soil inorganic pyrophosphatase plays in making the condensed phosphates available for plant uptake has been discussed previously. The use of inorganic P fertilizers, however, suffers some limitations which affect their efficiency for supplying P to plants, namely fixation by soil constituents. A proposed solution to this problem is to use organic or inorganic P compounds which when added to soils remain soluble in the soil solution and thus are not as subject to fixation. This is especially true when the P fertilizer must be broadcasted on the surface

of a soil as in a pasture, or applied on the soil surface by means of irrigation waters.

A study of the phosphatases in soil which are able to hydrolyze phosphates added to soils and the rates at which this hydrolysis occurs is important in assessing the effectiveness of using water-soluble P compounds as P fertilizers. Too rapid hydrolysis will not allow the P compounds to penetrate into the soil profile, because it is rapidly converted to Pi which becomes fixed and resists further movement. Slower hydrolysis is desirable because it allows the P compound to penetrate into the soil profile while still being able to supply Pi for plant uptake. However, compounds that remain soluble in the soil solution, but are not hydrolyzed, are not efficient P fertilizers because the P contained in them does not become available to plants. A study made by Pinck et al. (1941) showed that some organic phosphates such as triethyl and trimethyl phosphates could be completely recovered from two of three soils studied after three weeks of incubation under aerobic conditions at 25 C. The rate of hydrolysis of the water-soluble P compounds is thus a controlling factor in the process of determining the usefulness of such compounds as P fertilizers.

Important information needed to assess the usefulness of an organic P compound as a fertilizer is the P content of that compound. A high P content assures us that net mineralization will occur. The C:P ratio value at which mineralization will

begin to occur is given by Alexander (1977) as 200:1.

The mineralization (hydrolysis) process that occurs in the soil environment is largely controlled by phosphatases and a wide variety of phosphatases in soil have been demonstrated. Eivazi and Tabatabai (1977) using mono-, di- and tris-p-nitrophenyl phosphate as substrates found that soils contained phosphoric monoester hydrolases, phosphoric diester hydrolases, and phosphoric triester hydrolases. It is generally accepted that there exists, in soils, activity that will degrade almost any natural and most artificial substrates added to soils. Cosgrove (1967) states that "it is not possible to do other than draw the general conclusion that there exists in soil a wide range of microorganisms capable of dephosphorylating all known organic phosphates of plant origin."

The hydrolysis of organic P compounds added to soils by phosphatases is influenced greatly by pH. Phosphoric monoester hydrolase has been shown to have several optimal pH values. In an acid soil, the activity is made up almost entirely of acid phosphatase activity with a pH optimum of 6.5, while in a calcareous soil, the maximum activity is observed at pH 11 (Eivazi and Tabatabai, 1977). This pH effect is important because generally the closer the natural soil pH is to the pH optimum of the enzyme, the more soil activity will be observed. Thus, alkaline soils which contain high levels of alkaline phosphatase activity will not exhibit much activity under natural soil conditions because the soil

pH will not approach the pH optimum (11) needed for optimum activity to occur. Rolston et al. (1975) studied glycerophosphate as a potential source of phosphate and found that it moved greater distances into a column of a calcareous soil than did Pi. However, he found that the relative amount of movement of glycerophosphate, methyl ester phosphate, ethyl phosphate, glycol phosphate, and glucose-6-phosphate were similar to that of Pi in an acid soil.

Another important factor that governs the rate of hydrolysis of organic P added to soils is oxygen content, which in turn is related to moisture levels (e.g., aerobic vs. waterlogged). When a soil is submerged, the oxygen supply is cut off and reduction sets in. A result of reduction is an increase in the concentration of water-soluble and available P. A hundredfold increase in soluble P concentrations was observed in the water layer below the constant thermocline of a lake after reduction of the mud-water interface (Mortimer, 1941). The increased uptake of added P or native P by rice plants or by soil test extractants shows increased solubility of P with submerged soils (Shapiro, 1958; Broeshart et al., 1965; Mahapatra and Patrick, 1969; Patrick and Fontenot, 1976). The increase in concentration of water-soluble P when acid soils are submerged result from (1) hydrolysis of Fe(III) and Al phosphates, (2) release of P held by anion exchange on clay and hydrous oxides of Fe(III) and Al, and (3) reduction of Fe(III) to Fe(II) with liberation of sorbed and chemically

bonded P. In alkaline soils, the increases in soluble P concentrations are due to decreases of pH upon flooding, which causes greater solubility of hydroxyapatite (see Ponnamperma, 1972). This increased concentration of soluble P, primarily as Pi, is important because Pi has been shown to be a competitive inhibitor of phosphatases in soils (Browman and Tabatabai, 1978; Juma and Tabatabai, 1978).

Sources of Phosphatases in the Soil Environment

The role accumulated enzymes play in the P cycle of soils has been the object of an increasing amount of study but some basic questions concerning phosphatases, and enzymes in general, in the soil environment still remain unanswered. How are enzymes released and accumulated in soil? What are the sources of accumulated enzymes in soil? This will be the subject of discussion in this section. A further question is how are these accumulated enzymes stabilized in the soil and thus become protected from inactivation by extremes of heat-cold, wet-dry, and from proteolytic enzymes stabilized in the soil and exuded from soil microorganisms. This topic will be discussed in the next section of this review.

Ramirez-Martinez and McLaren (1966) reported that a tremendous amount of enzyme protein accumulation seems to occur in soils. The amount of phosphatase activity usually found in 1 g soil is equivalent to that produced by huge numbers of microorganisms. The amounts of microorganisms calculated

to give an equivalent activity were 10^{10} bacteria or 1 g of fungal mycelia. Assuming that nonprotein soil components do not catalyze hydrolysis of phosphorus compounds in soils, we must assume that the extracellular components of phosphatase activity in soils must originate from living tissue. Both plants and microorganisms can release phosphatases into the soil environment. Ribonucleases and alkaline phosphatase are excreted by Bacillus subtilus under certain conditions (Nishimura and Nomura, 1959; Cashel and Freese, 1964) and pyrophosphatase and acid phosphatase may exist extracellularly on the surface of cell walls of Saccharomyces mellis by Weimberg and Orton (1963, 1964). Jacquet et al. (1956) also showed that a number of bacteria release phosphatases. Spier and Ross (1978), in their review of soil phosphatases, suggest that microorganisms seem the logical choice for supplying most of the soil enzyme activity because of their large biomass, high metabolic activity, and short lifetimes which allow them to produce and release a relatively large amount of extracellular enzymes. The effect of microorganisms in supplying phosphatase activity to soils, however, seems temporary and short-lived. Ladd and Paul (1973) incubated a soil with glucose and sodium nitrate at 22 C and found that bacterial numbers increased almost 2-fold in 36 hours, which was accompanied by an increase of 3.2-fold in phosphatase activity. However, the activity was rapidly lost, and after 21 days had completely disappeared. Spier and Ross (1978) theorized that the in-

creased activity of phosphatase produced during incubation of soil with glucose and nitrate was largely lost during the microorganisms cycle of proliferation-dying-lysing, and that many flushes of microbial activity may be required to obtain a permanent increase in the extracellular level of phosphatase activity.

Plants have also been considered to be a source of extracellular enzymes in soil. Estermann and McLaren (1961) using barley as the test plant found that root caps possessed phosphatase activity. Later, Juma (1976) demonstrated acid phosphatase activity in sterile corn and soybean roots but no alkaline phosphatase activity was evident. He further demonstrated that sterile corn and soybean roots could exude phosphatases into a solution which surrounded them. Roots were placed into sterile buffer or water and the solution tested for phosphatase activity after 4-48 hours. The amount of phosphatase released from the roots was dependent on whether they were suspended in water or the buffered solution, with greater amounts of phosphatase being released into water than the buffered solution.

The type of vegetation and thus the type of organic matter (or litter) added to a soil has a great effect on soil enzyme activity. Plants are able to synthesize many enzymes found in soils, which may remain active for some time after being returned to the soil. Spier (1976) and Spier and Ross (1976) found that the phosphatase activity of green tussock

leaves was much higher, on a dry weight basis, than that found in soils but that the activity declined with increasing age. However, they were not able to determine whether phosphatase activity of the fallen litter was due to residual plant enzyme or to microorganisms decomposing the litter. Spier (1976) concluded that the roots of tussock were a much more likely source of phosphatase in soils and that these roots could significantly add to soil phosphatase activity.

Plants influence phosphatase activity by indirect means also. Many investigators have shown that enzyme activity is considerably greater in the rhizosphere of plants than in soils and this increased activity could be due to either a specific flora, the plant root, or both (see Skujins, 1967). Another indirect influence plants have on enzyme activity is the increased number of microorganisms present upon addition of plant litter to soils. This effect has already been discussed under the role microorganisms play in supplying enzyme activities to soils.

State of Enzymes in Soils

The term "state of enzymes in soils" has been used by Skujins (1967, 1976) to describe the phenomena whereby enzymes are stabilized in soils. Describing the state of the enzyme in the soil is to attempt to give the location and microenvironment in which it functions, and how the enzyme is bound or stabilized within that microenvironment.

The stability of enzymes within the soil matrix has resulted in several theories being proposed to explain the protective influence of the soil on enzymatic activity. The dominant mechanisms of enzyme immobilization have been summarized by Weetall (1975) and are shown in Figure 1a reproduced from Burns (1978). These mechanisms have all been suggested to explain the immobilization and protection of enzymes in the soil environment.

Ensminger and Gieseking (1942) reported that protein adsorbed to montmorillonite was stabilized against microbial attack. These results gave support to the concept that the most important factor in enzyme stabilization was the clay fraction of the soil. A study by Haig (1955) supported this hypothesis. He found that acetyl-esterase activity in a fine sandy loam soil that had been fractionated into sand, silt, and clay sizes, was associated primarily with the clay fraction. McLaren (1954b) observed that kaolinite adsorbed trypsin and chymotrypsin. This adsorption occurred rapidly, being nearly complete after 2 to 3 min. McLaren (1954b) also found that heat inactivated lysozyme in solution and bound to kaolinite was hydrolyzed by chymotrypsin but the rate of hydrolysis was slower for the lysozyme adsorbed to the kaolinite. Adsorption of pepsin and lysozyme by Mg-bentonite was studied by Armstrong and Chesters (1964) and they also reported that the adsorption of protein was rapid, with 90% occurring within 3 min. The mechanism of binding of proteins

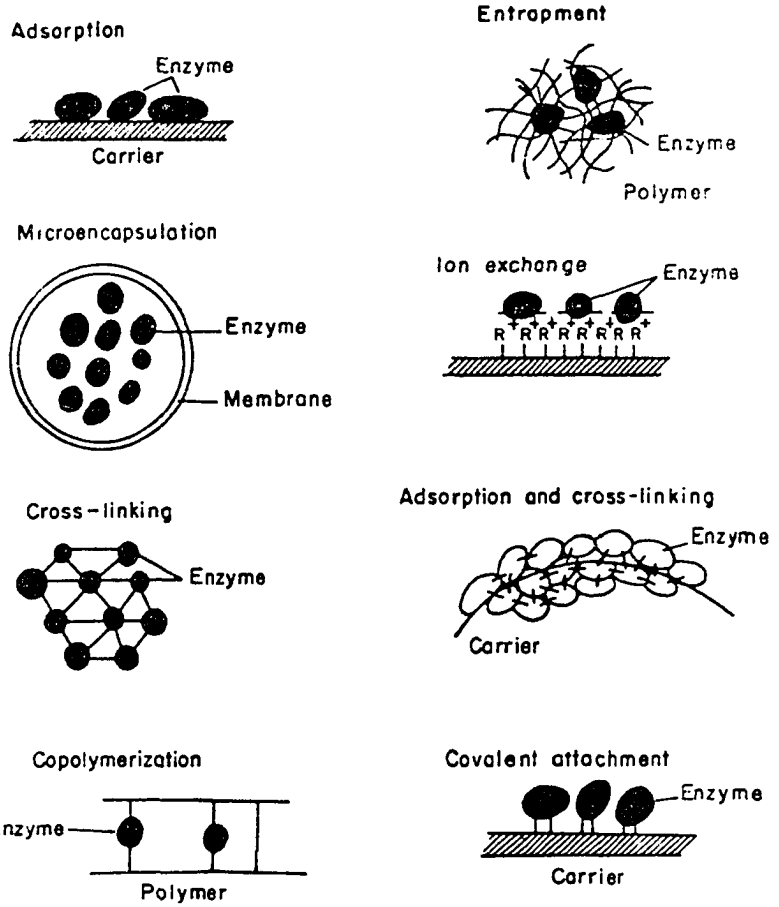


Figure 1a. Schematic representation of methods of immobilizing enzymes (Weetall, 1975)

by clay minerals has not yet been determined, but Albert and Harter (1973) reported that adsorption of lysozyme and ovalbumin by Na-clay minerals caused an increase in sodium ion concentration of the clay-protein suspension. They interpreted this result as evidence that a cation exchange adsorption mechanism was occurring.

Stabilization of enzymes in the soil environment by soil organic matter rather than soil inorganic components has also been suggested. Much of the information dealing with this hypothesis has been gathered by studies involving synthetic polymer-enzyme complexes (for a review of this topic, see Ladd and Butler, 1975). In a study by Burns et al. (1972b), it was found that an organic fraction extracted from soil, which was free of clays (confirmed by x-ray analyses), contained urease activity. Burns et al. (1972b) also found that urease adsorbed by bentonite was degraded by pronase added to the system, but urease bound in a bentonite-urease-lignin complex was resistant to proteolytic attack by the pronase.

Ladd and Butler (1975) suggested that the enzyme protein of a humus-protein complex could be bound by hydrogen, ionic, or covalent bonding to soil humus. The extent that enzymes are bound by each of these mechanisms is difficult to determine. Work by Simonart et al. (1967) suggests that hydrogen bonding may be only a minor factor in enzyme stabilization in soils. Using phenol as a hydrogen bond breaking solvent, they were able to dissolve only a small amount of proteinaceous

material.

Ionic bonding also seems to play a role in enzyme stabilization, and such a mechanism for binding of enzyme to soil organic matter was proposed by Butler and Ladd (1969). They proposed that the organic matter binds enzymes by amino-carboxyl salt linkages. Such complexes, however, should be easily broken by many of the extraction reagents (i.e., urea and pyrophosphate) used to remove active enzyme materials from soil. However, the small yields of active enzyme materials that have been extracted from soil to date indicates that mechanisms which propose ionic bonding may not be entirely valid (Hayano, 1977; Hayano and Katani, 1977; Ceccanti et al., 1978). However, Burns et al. (1972a) extracted approximately 20% of the original soil urease activity by using urea (urea being hydrolyzed subsequently by the extracted urease). The clay-free precipitation obtained contained urease activity which was not destroyed by the addition of the proteolytic enzyme, pronase. Burns et al. concluded that native soil urease is located in organic colloidal particles which contain pores large enough for water, urea, ammonia, and carbon di-oxide to pass freely, but small enough to exclude pronase.

A clear hypothesis of enzyme immobilization by means of covalent attachment has not yet been described. Ladd and Butler (1975) suggested that linkage of soil quinones by nucleophilic substitution to sulfhydryl and to terminal and ϵ-amino groups of enzyme proteins may lead to active organo-

enzyme derivatives, provided that these groups do not form a part of the active site of the enzyme.

A hypothesis that has received little attention to date is that the enzymes in soils are glycoproteins. Malathion esterase extracted from soil by Satyanarayana and Getzin (1973) was thought to be a glycoprotein based on the following evidence: (1) amino acids constituted only 65% of the purified enzyme and (2) a carbohydrate splitting enzyme, hyaluronidase, enhanced the catalytic effect of the esterase, presumably by loosening the carbohydrate shield and allowing the protein core to gain easier access to the substrate. The evidence gained by incubating the esterase with hyaluronidase suggested that the carbohydrate-protein linkage occurs through N-acetylhexoseamine-tyrosine bonds. Mayaudon et al. (1975) concluded similarly, when they observed that diphenol oxidase activity was not affected by pronase alone, but was destroyed when incubated in the presence of both lysozyme and pronase.

In soils, a strong association between clay and humus exists and McLaren (1975) postulated that soil enzymes become incorporated in a three-dimensional network of clay and humus complexes. Burns et al. (1972b) reported that bentonite alone did not protect urease from pronase attack but that a bentonite-lignin complex did. They concluded that only soil organic matter operates in enzyme stabilization. However, no evidence was provided to show that lignin alone protected urease from pronase attack.

Influence of Clay Minerals on Enzyme Activity

Clay minerals may play an important part in soil enzyme stabilization as has been previously suggested. This hypothesis is based on the observation that adsorption of proteins by clays has been observed (Ladd and Butler, 1975). Clays exhibit strong sorptive qualities because of their highly charged surfaces. Studies of esterase and urease activities in soil have shown that the highest activity was associated with the clay fraction of the soil, with very little being associated in the sand fraction (see Skujins, 1976).

Adsorption of enzyme proteins by clay minerals appears to result in loss of activity (McLaren, 1954a; Ross and McNeilly, 1972; Makboul and Ottow, 1979a,b,c). Ensminger and Gieseking (1939, 1942) and McLaren (1954a) studied the adsorption of proteins unto clays and found that adsorption occurs over a wide pH range, and rather stable clay-protein complexes are formed. The adsorption reaction was reversible, however, and could be desorbed with a minimal loss in activity (McLaren, 1954a; McLaren and Estermann, 1956). Harter and Stotzky (1971) found no correlation between suspension pH and protein adsorption. Instead, protein molecular weight and the cation on the clay exchange complex appeared to be the controlling factor. Albert and Harter (1973), however, pointed out that the conclusion that pH did not have any effect on protein adsorption was based on the suspension pH of clays

containing different ions, and not on different suspension pH values of one homoionic clay. In a further study of the effect of pH on sorption of lysozyme and ovalbumin by clay minerals, Albert and Harter (1973) found that protein adsorption was influenced by clay suspension pH, with adsorption increasing as the pH approached the protein isoelectric point. Smectite and biotite-vermiculite adsorbed protein to a much greater degree than did kaolinite and illite. They concluded that the excess adsorption by smectite and biotite-vermiculite was evidence that interlayer entrapment of protein occurred.

A recent study of arylsulfase adsorption by montmorillonite was undertaken by Simpson and Hughes (1978). Using x-ray diffraction techniques, they observed an increase in the interlattice spacing of 9 Å for the dehydrated arylsulfatase-montmorillonite complex over that of the montmorillonite clay alone. However, a molecular diameter of 50 Å was estimated for arylsulfatase enzyme which is far too large to fit into the 9 Å spacing. They suggested that bonding of arylsulfatase to montmorillonite caused denaturation of the enzyme protein resulting in the formation of a polypeptide chain approaching a diameter of 4.3 Å. Thus, the observed interlattice spacing increase of 9 Å was considered to be due to a duo-layer adsorption of denatured arylsulfatase polypeptide chains within the interlayer clay lattice.

Recently, Makboul and Ottow (1979a,b,c) reported that alkaline phosphatase, urease, and acid phosphatase were

inhibited by clay minerals. Increasing concentrations of montmorillonite, illite and kaolinite in Ca-form all decreased the V_{max} values while increasing the K_m values of urease. For alkaline phosphatase, montmorillonite and kaolinite did not significantly affect the V_{max} values while the K_m values increased with increasing clay concentrations. Increasing amounts of illite, however, showed a significant lowering of the V_{max} values while the K_m values remained relatively constant. Tabatabai (Department of Agronomy, Iowa State University, personal communication) replotted these data using the Lineweaver-Burk reciprocal plot technique and concluded that the inhibition by kaolinite and montmorillonite showed competitive kinetics while illite showed noncompetitive kinetics. For acid phosphatase, increasing levels of clay in the assay system decreased the K_m value, but the V_{max} values remained relatively constant at the different concentrations of kaolinite and illite used. However, the V_{max} value for acid phosphatase increased markedly when increasing amounts of montmorillonite were added to the assay system.

The inhibitory effect of allophane and montmorillonite on protease, alpha-amylase, and beta-amylase prepared from Streptomyces griseus, bacteria (not specified), and barley, respectively, was reported by Kobayashi and Aomine (1967). They found that montmorillonite had a greater effect on protease and beta-amylase than did allophane, while the reverse was the case for alpha-amylase. The K_m and V_{max} values were

reduced when protease and alpha-amylase were adsorbed by allophane and montmorillonite. Adsorption was thought to increase the formation of an enzyme-substrate complex while simultaneously reducing the velocity of the breakdown of the complex to form product. For beta-amylase an increase in the K_m value and a decrease in the V_{max} value occurred when it was adsorbed by allophane. Thus, allophane was considered to be both a partially competitive and a partially noncompetitive inhibitor.

Although clays have been shown to inhibit enzyme activity in many instances outside of the soil environment, Kroll and Kramer (1955) showed that montmorillonite added to soil did not affect the activity of phosphatase. This is not surprising if we realize that the amount of clay in soil is already sufficient to adsorb all the enzyme present and only minute amounts of free enzymes are found in soils.

This study was made to evaluate some of the points discussed in this review. How is soil pyrophosphatase affected by metal ions? What are the relative rates of hydrolysis of various organic and inorganic P compounds added to soils and are these rates related to soil phosphatase activity? Can plants, manures and sewage sludges contribute to phosphatase activity in the soil environment? And, finally, how do clay minerals affect the activity of acid phosphatase and pyrophosphatase derived from plant roots?

PART I. EFFECTS OF METAL IONS ON INORGANIC PYROPHOSPHATASE ACTIVITY OF SOILS

INTRODUCTION

The increased use of condensed phosphates as fertilizers has stimulated much interest in their behavior in soils. About half of the phosphorus in ammonium polyphosphate fertilizer is in the orthophosphate form (Pi). The other half is in condensed forms, primarily as pyrophosphate (PPi) (Hashimoto and Lehr, 1973). The value of PPi as a fertilizer source of phosphorus is dependent on its rate of hydrolysis to Pi (Sutton et al., 1966). Hydrolysis of PPi in soils is brought about almost exclusively by enzyme catalysis, with pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) being the primary enzyme responsible for this reaction (Dick and Tabatabai, 1978). Until recently, the information on the catalysis brought about by this enzyme in soils was mainly derived from studies of extraction of Pi from soils after incubation with PPi (Gilliam and Sample, 1968; Sutton et al., 1966). Three problems are associated with the measurement of Pi released by enzymatic hydrolysis during assay for pyrophosphatase: (1) the Pi released may be sorbed by soil constituents and therefore not extracted, (2) Pi may continue to be hydrolyzed from PPi after extraction from the soil for reasons other than the enzymes (e.g., low pH), and (3) the presence of PPi may interfere with the measurement of Pi. Recently, Dick and Tabatabai (1978) developed a method for assay of inorganic pyrophosphatase activity in soils that

overcomes these problems. This method is simple and precise and is based on the extraction and colorimetric determination of Pi released when 1 g soil is incubated with buffered (pH 8) PPi solution at 37 C for 5 h.

Numerous investigators have reported the absolute requirement for divalent cations by pyrophosphatase, purified from various biological sources, for activity to occur (Josse and Wong, 1971; Howard and Lundgren, 1970; Butler, 1971). Butler and Sperow (1977) have shown that yeast pyrophosphatase has three roles for metal ions in its reaction: activator, substrate, and structural. The three roles are independent and individual metal ions can satisfy only one role at a time. The metal ions specificity for the three roles declines in the order: structural > substrate > activator. Out of a wide variety of metal ions tested (Butler and Sperow, 1977) only Mg^{2+}, Zn^{2+}, Mn^{2+}, and Co^{2+} were found to fulfill both the activator and substrate roles. Butler (1971) showed that the relative activity of pyrophosphatase purified from yeast decreased upon addition of divalent cations in the following order: $Mg^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} >> Ca^{2+}$. Also, Naganna and Menon (1948) and Josse (1966) reported strong inhibition by Ca^{2+} of Mg^{2+}-stimulated pyrophosphatase purified from erythrocyte cells and Escherichia coli, respectively.

The effect of metal ions on pyrophosphatase activity of a mixed enzyme system extracted from microorganisms isolated from soil was studied by Searle and Hughes (1977). They

found that the specific activities at the optimum pH and cation concentration decreased in the following order: Mg^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Fe^{2+}. The influence of Mg^{2+} and Ca^{2+} on soil pyrophosphatase was recently investigated by Tabatabai and Dick (1979). They found that the mole fraction of water soluble Mg/(Mg + Ca) was highly correlated (r = 0.78**) with the log of pyrophosphatase activity of 18 Iowa surface soils. In addition, Dick and Tabatabai (1979) observed that in the 5 calcareous soils used, the log of pyrophosphatase activity was significantly but negatively correlated (r = -0.97**) with $CaCO_3$ equivalent. The decrease in activity was considered to be due to binding, by the carbonate, of the Mg^{2+} required for activation of PPi so that pyrophosphatase activity can occur (Butler, 1971). Little information is available, however, on the degree of activation or inhibition of pyrophosphatase in soils by other metal ions. In this study, three soils were leached with ammonium acetate to remove the soluble metal salts and exchangeable metal ions. The effect of various added metal ions on the activity of pyrophosphatase was studied using a range of metal ion concentrations.

DESCRIPTION OF METHODS

The soils used in this investigation are described in Table 1. In the analyses reported, pH was determined by a glass electrode (soil:water ratio, 1:2.5), organic C by the method of Mebius (1960), particle-size distribution by the pipette analysis of Kilmer and Alexander (1949), and pyrophosphatase activity was assayed by the method of Dick and Tabatabai (1978). The soils were leached with NH_4OAc to remove the soluble salts and exchangeable cations as described by Chapman (1965). In this procedure, 6 portions of soil, each containing 50 g, were mixed and equilibrated with 500 ml 1 N NH_4OAc (pH 8) for 24 h, filtered (55-mm Buchner funnel, #42 Whatman filter paper), and leached with additional 25 ml increments of the NH_4OAc reagent (pH 8) until a negative test for calcium was obtained. Then the soil was leached 4 times with N NH_4Cl (pH 8) using 50 ml increments and once with 0.25 N NH_4Cl (pH 8), and the excess electrolyte washed out with 200 ml of 99% isopropyl alcohol. After leaching, the soils were air-dried and ground to pass a 100-mesh sieve. Calcareous soils were not included in this study because of the difficulty in removing calcium carbonate and exchangeable Ca^{2+} by the procedure described.

All metal ions used were chloride salts, except Fe^{2+} and Co^{2+} which were sulfate salts, and were first prepared as stock solutions before being diluted as required to supply the

Table 1. Properties of soils

Soil type	pH	Organic C (%)	Clay (%)	Sand (%)	Pyrophosphatase activity[a]
Clarion l	4.6	1.99	24	37	10.8 (1.94)
Nicollet cl	6.2	2.73	29	34	13.8 (4.32)
Webster l	6.5	2.91	26	37	12.7 (6.43)
Okoboji cl	7.0	5.32	36	21	7.3

[a] μmoles Pi released·g soil^{-1}·5 h^{-1}. Figures in parentheses are those obtained after leaching soils with NH_4OAc.

necessary metal ion to the assay mixture. Assays designed for testing the effect of various metal ions on soil pyrophosphatase activity were performed by weighing 1 g of the NH_4OAc-leached soil into a 50-ml plastic centrifuge tube. The soil was treated with 1 ml of the metal ion solution and 2 ml of universal buffer (pH 8), prepared as described by Skujins et al. (1962), containing tetrasodium pyrophosphate so that the final concentrations of metal ions and PPi were 0, 10, 20, 35, 50, or 70 m$\underline{M}$ and 50 m$\underline{M}$, respectively (expressed on a soil basis, the metal ion concentrations used were 0, 30, 60, 105, 150, and 210 μmole/g soil). The samples were incubated for 5 h at 37 C, removed from the incubator, and treated with 2 ml of universal buffer (pH 8). Next they were treated with 25 ml $\underline{N}$ H_2SO_4, stoppered, shaken on a mechanical shaker for 3 min, centrifuged (1 min at 10,000 rpm), and a

1-ml aliquot was analyzed for Pi as described by Dick and Tabatabai (1978). Controls were performed by incubating the soil samples for 5 h with 1 ml metal ions solution and 2 ml universal buffer (pH 8). After incubation, 2 ml PPi solution were added, the soil extracted with $\underline{N}$ H_2SO_4, and a 1-ml aliquot analyzed for Pi as described above. The increase in Pi content in the assay sample over that of the control sample was taken as a measure of pyrophosphatase activity. The effect of Ca^{2+} and Mg^{2+} on pyrophosphatase activity of soils was further investigated by making assay measurements at 4 constant PPi concentrations (50, 75, 100, and 150 m$\underline{M}$), while increasing the Ca^{2+} and Mg^{2+} concentrations from 0 to 250 m$\underline{M}$ (0-750 μmole/g soil) at each of the PPi concentrations indicated.

In studies of effect of temperature on pyrophosphatase activity, field-moist and air-dried soil samples (not subjected to the NH_4OAc-leaching procedure described above) were incubated 1 h at temperatures ranging from 30-120 C. The samples were than brought to 22 C before assay of pyrophosphatase activity at 37 C (Dick and Tabatabai, 1978). The effect of Ca^{2+} and Mg^{2+} on heat inactivation of soil pyrophosphatase was tested by treating 1 g NH_4OAc-leached soil with 0.5 ml water, 200 m$\underline{M}$ $CaCl_2$, or 200 m$\underline{M}$ $MgCl_2$. The samples were heated at 80 or 90 C for 0, 5, 10, 15, 20, 25, or 30 min, cooled to room temperature, and 0.5 ml of water was added to the samples treated with 0.5 ml of 200 m$\underline{M}$ $CaCl_2$ or $MgCl_2$. The controls

(samples treated with 0.5 ml of water) were treated with 0.5 ml of 200 mM $CaCl_2$ or $MgCl_2$. Then 2 ml of universal buffer containing PPi were added to give 50 mM PPi, and the samples assayed as described previously.

Since previous work showed that Na_2CO_3 inhibits pyrophosphatase activity, perhaps by precipitating Ca^{2+} and/or Mg^{2+}, the effect of addition of Ca^{2+} or Mg^{2+} to overcome this inhibition in Na_2CO_3-treated, NH_4OAc-leached soils was studied. In this test, 1 g of the NH_4OAc-leached soils was treated with 0.5 ml Na_2CO_3 and 0.5 ml $CaCl_2$ or $MgCl_2$ solutions, and 2 ml of universal buffer (pH 8) containing PPi. The final concentration of Na_2CO_3 in the assay mixture was 0, 10, or 50 mM, the final concentration of $CaCl_2$ or $MgCl_2$ was 0, 20, 35, 50, 70, or 100 mM, and the final concentration of PPi was 50 mM.

In the work reported, all values are averages of duplicate analyses of pyrophosphatase activity and are given on an oven-dry basis, soil being dried at 105 C for 36 h.

RESULTS AND DISCUSSION

Results obtained when the NH_4OAc-leached soils were assayed for pyrophosphatase activity showed that much less activity was observed in the NH_4OAc-leached soils than in soils not leached with NH_4OAc (Table 1). Much of the pyrophosphatase activity may have been removed from the soils during the NH_4OAc leaching procedure, since the leachates obtained by this procedure were light yellow in color indicating that some organic matter was extracted. The effect of isopropyl alcohol on pyrophosphatase activity may also have caused a change in the activity observed in the NH_4OAc-leached soils. Lethbridge et al. (1976) investigated the effect of organic solvents on soil urease activity and found that acetone and hexane decreased activity, and allowing time (5 h) for evaporation of the solvents to occur did not change their inhibitory effect. In the work described here, the isopropyl alcohol used in leaching out the excess electrolyte from the soils treated with NH_4OAc may have caused the decrease observed in pyrophosphatase activity. However, interpretation of the results obtained when the NH_4OAc-leached soils were assayed for pyrophosphatase activity in the presence of metal ions would not change because their effect on the pyrophosphatase still active would not change. The effect that NH_4^+ has on decreasing pyrophosphatase activity was also considered. Dick and Tabatabai (1978) found, however, that addition of

50 μmoles/g soil of NH_4^+ as NH_4Cl, did not cause any change in the level of pyrophosphatase activity observed in soils.

The hydrolysis of inorganic pyrophosphate (PPi) by pyrophosphatase occurs only in the presence of divalent metal ions (Searle and Hughes, 1977; Kunitz, 1952). The metal ions are necessary to fulfill three different and independent roles (Butler and Sperow, 1977): (1) free metal ions activate the enzyme, (2) metal-PPi is the actual substrate which is bound via the metal ion to the enzyme rather than through the phosphate group, and (3) metal ions play a role in keeping the enzyme in its proper three-dimensional form for activity to occur.

In the experiment in which monovalent and divalent metal ions were added to the NH_4OAc-leached soils before assay of pyrophosphatase activity, the metal ions may serve two roles: the activating role, substrate role, or both. The results show that at certain concentrations Mg^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} promote, K^+ and Na^+ did not affect, and Fe^{2+} and Cu^{2+} inhibited soil pyrophosphatase activity (Figures 1b-3). Comparison of these results with those obtained by Searle and Hughes (1977) for pyrophosphatase obtained from cultures of soil microorganisms showed only slight differences. Searle and Hughes found that Fe^{2+} activated and Ca^{2+} had no effect on microbial pyrophosphatase, but they did not test the effect of Ba^{2+}, K^+, Na^+, and Ni^{2+}. Using three soils, the two most efficient promoters of soil pyrophosphatase were

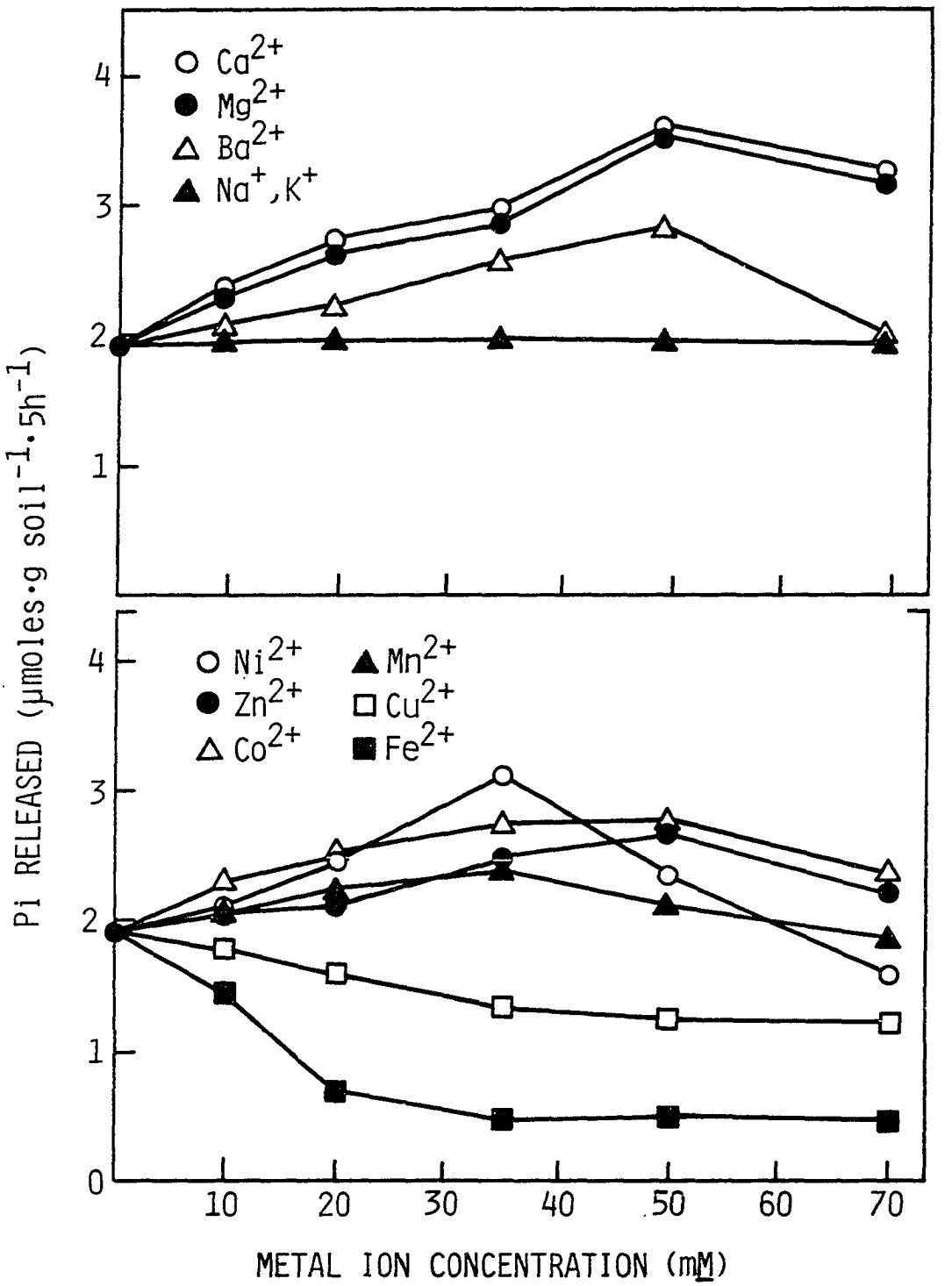


Figure 1b. Effects of metal ions and metal ion concentrations on pyrophosphatase activity in NH_4OAc-leached Clarion soil

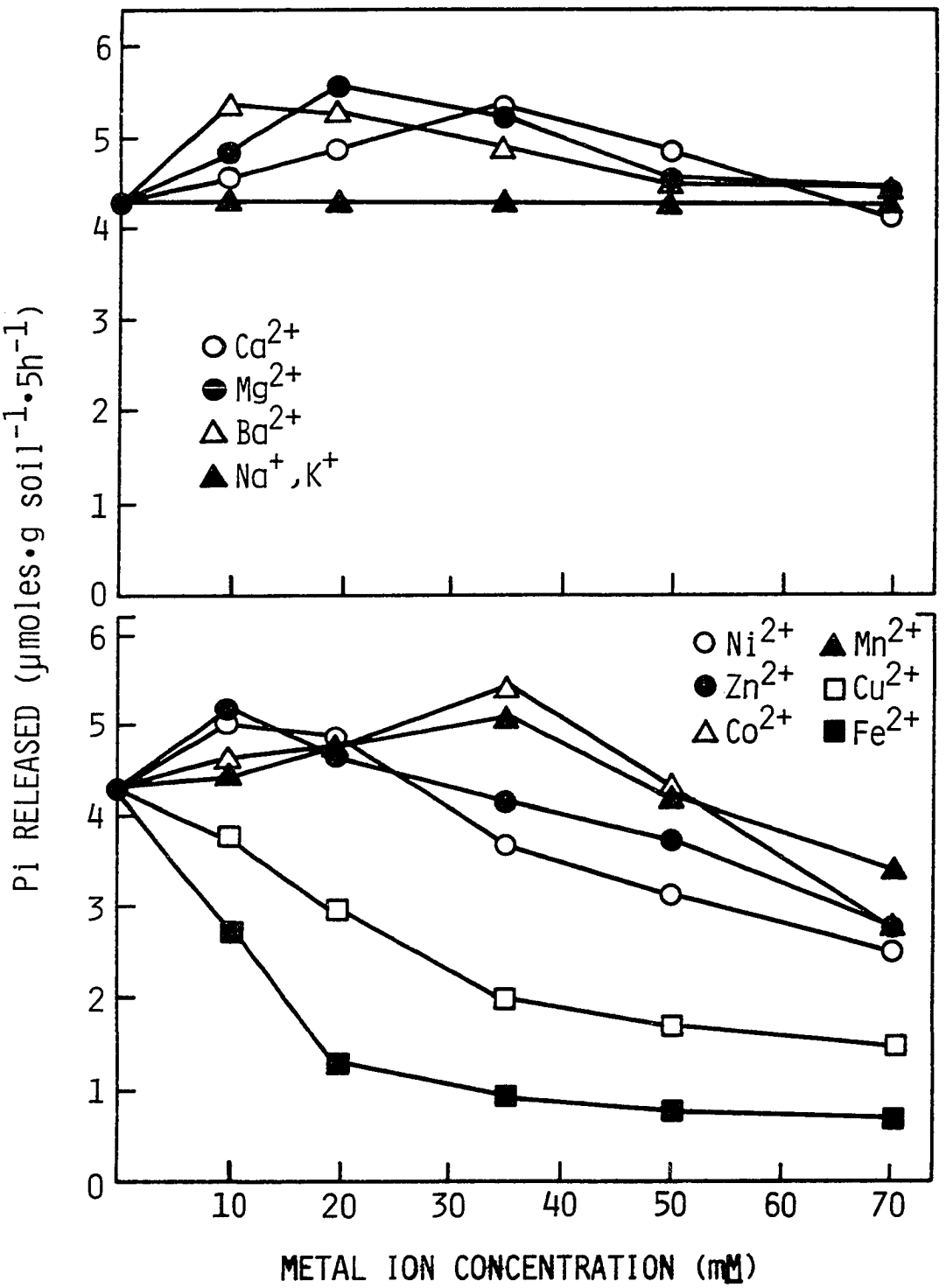


Figure 2. Effects of metal ions and metal ion concentrations on pyrophosphatase activity in NH_4OAc-leached Nicollet soil

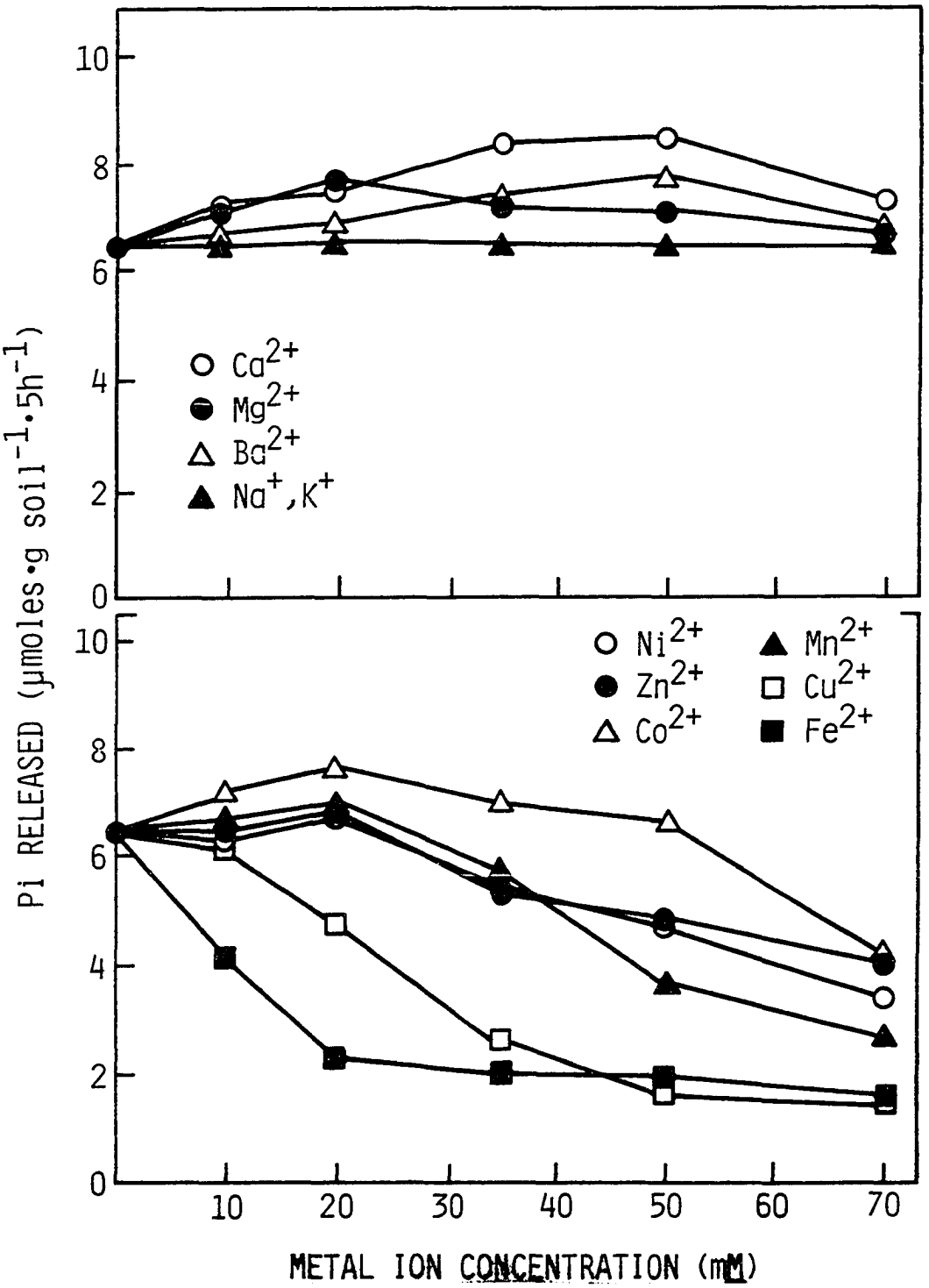


Figure 3. Effects of metal ions and metal ion concentrations on pyrophosphatase activity in NH_4OAc-leached Webster soil

Ca^{2+} and Mg^{2+} which increased the activity by an average of 47% and 42%, respectively, at the optimum metal ion concentration (Table 2). The optimum metal ion concentrations needed to promote maximum activity varied according to the metal ion and soil tested (Figures 1b-3). Nicollet and Webster soils generally reached their optimum activities at lower metal ion concentrations than did Clarion soil. With the average percentage increase in pyrophosphatase activity of the three soils in parentheses, the efficiency of the metal ions in promoting soil pyrophosphatase activity in decreasing order were: Ca^{2+} (47%) > Mg^{2+} (42%) > Ba^{2+} (29%) = Co^{2+} (29%) > Ni^{2+} (27%) > Zn^{2+} (20%) > Mn^{2+} (16%). The order of efficiency of these metal ions in promoting the activity in each soil, however, varied among the three soils tested (Table 2). It is interesting to note that the two metal ions (Ca^{2+} and Mg^{2+}) that best promoted pyrophosphatase activity are those most abundant in soils among the metal ions studied (Table 2).

Information of the effect of metal ions on promoting pyrophosphatase activity indicates that several different types of pyrophosphatases may be found in nature. The large majority of pyrophosphatases, however, seem to require Mg^{2+} for optimum activity (Butler, 1971). One of the most widely studied pyrophosphatases has been isolated from baker's yeast, and studies with this enzyme have shown that other metal ions are poor substitutes for Mg^{2+} in promoting its activity (Kunitz,

Table 2. The effect of metal ions on pyrophosphatase activity in NH_4OAc-leached soils

Metal ion	Optimum pyrophosphatase activity in soil specified[a]			Average percentage increase
	Clarion	Nicollet	Webster	
None	1.94	4.32	6.43	
Ca^{2+}	3.59 (85)	5.36 (24)	8.49 (32)	47
Mg^{2+}	3.47 (79)	5.53 (28)	7.72 (20)	42
Ba^{2+}	2.79 (44)	5.36 (24)	7.72 (20)	29
Co^{2+}	2.74 (41)	5.53 (28)	7.59 (18)	29
Ni^{2+}	3.08 (59)	5.01 (16)	6.75 (5)	27
Zn^{2+}	2.64 (36)	5.14 (19)	6.82 (6)	20
Mn^{2+}	2.35 (21)	5.10 (18)	6.94 (8)	16

[a] μmoles Pi released·g soil^{-1}·5 h^{-1}. Figures in parentheses are percentage increase in activity in the presence of metal ion relative to that obtained in the absence of metal ions (Figures 1b-3).

1952). Butler and Sperow (1977) reported that only Mg^{2+}, Zn^{2+}, Mn^{2+}, and Co^{2+} are able to fulfill both the activator and substrate roles of yeast pyrophosphatase enzyme. However, Mg^{2+} is the only metal known to fulfill the structural role as well.

Levinson et al. (1958) reported that pyrophosphatase activity from B. megaterium spores was mainly promoted by Mn^{2+}, and to a much lesser extent by Zn^{2+} and Mg^{2+}. Other studies have shown that Mn^{2+} is the main promoter of pyrophos-

phatase activity in the vegetative cells and spores of B. megaterium and B. subtilis, but Mg^{2+}, Ca^{2+}, Zn^{2+}, and Co^{2+} also promoted the activity of this enzyme (Tono and Kornberg, 1967a, b). A pyrophosphatase purified from human erythrocyte cells (Pynes and Younathan, 1967) has properties very similar to those of the pyrophosphatase isolated from baker's yeast (Butler, 1971), but it has much greater substrate specificity in the presence of Zn^{2+}. An unusual pyrophosphatase purified from Streptococcus faecalis was described by Oginski and Rumbaugh (1955). Its activity was promoted by Co^{2+} and it had optimum activity near pH 5, which is much lower than that normally found for inorganic pyrophosphatase isolated from other biological sources.

The relatively large number of metal ions found to promote soil pyrophosphatase activity and the lack of any one metal ion to give a single large increase in activity suggests that either there are a number of pyrophosphatases in soil or that the effectiveness of some of the metal ions studied is influenced by their sorption by the soil constituents. This is in agreement with the conclusion of Searle and Hughes (1977), that diverse microorganisms produce only one "functional" kind of enzyme, which can be promoted by a range of metal ions. In their work, however, the microorganisms were isolated from one soil on a single medium.

Another explanation for the lack of a single promoter of great efficiency is that pyrophosphatases that exhibit acidic

pH optima are less specific than corresponding enzymes with neutral or alkaline pH optima and have little or no requirement for divalent cations (Brightwell and Tappel, 1968). McLaren and Estermann (1957) have shown that the pH optimum in soils is generally 1-2 pH units higher than in solution. This is due to the differential pH which exists between the clay and humus surfaces and the bulk solution. Since the measured pH optimum for soil pyrophosphatase is 8 (Dick and Tabatabai, 1978), it is possible that the actual pH optimum at the soil-solution interface is near 6.

Our observation that Ca^{2+} was the most effective promoter of pyrophosphatase activity is somewhat unique. In most studies, Ca^{2+} has been found to have no effect in promoting pyrophosphatase activity and strongly inhibits the activity promoted by Mg^{2+} (Kunitz, 1952; Butler and Sperow, 1977). However, in fulfilling the activator role for the enzyme, there seems to be little difference which metal ion is present (Butler and Sperow, 1977). The substrate role of the metal ion is fulfilled by first binding to PPi to produce a metal ion-PPi complex. This complex is considered the actual substrate for the pyrophosphatase enzyme and it binds to the enzyme through the metal ion rather than the phosphate groups. Thus, the metal ion bound to PPi to form the metal ion-PPi complex can exert a great influence on pyrophosphatase activity. For yeast inorganic pyrophosphatase, both CaPPi and MgPPi interact with the Mg^{2+}-activated enzyme but MgPPi is

hydrolyzed 10^5 times faster, although CaPPi bonds about 50 times more strongly than MgPPi to the enzyme (Butler, 1971). The effect Ca^{2+} exerts in promoting soil pyrophosphatase activity seems to be due to its interaction with the enzyme itself. No observable chemical hydrolysis occurred when Ca^{2+}, or the other metal ions studied, and PPi were incubated together without soil for 5 h. Butler and Sperow (1977) also observed that the divalent metal ions that they tested did not cause any appreciable chemical hydrolysis of PPi.

The finding that Fe^{2+} inhibits soil pyrophosphatase activity contradicts the results reported by Searle and Hughes (1977), who found that promotion of pyrophosphatase activity occurred only when N_2 was bubbled through the assay mixture to insure keeping Fe^{2+} in the reduced state. N_2 was not used in the work described, and perhaps Fe^{2+} was oxidized to Fe^{3+} during the assay conditions described.

Since metal ions are required for pyrophosphatase activity, the ratios of Mg^{2+}:PPi and Ca^{2+}:PPi on the optimum activity of this enzyme in two soils were studied. In these experiments, the pyrophosphatase in NH_4OAc-leached soils was assayed at four PPi concentrations, each containing increasing amounts of Mg^{2+} or Ca^{2+} (Figures 4 and 5). The results obtained showed that as the PPi concentrations increased, the concentration of metal ions required to achieve the optimum activity also increased. Also, the promoting effect of Mg^{2+} and Ca^{2+} was more apparent at the higher PPi concentrations.

Figure 4. Effects of Mg^{2+} and Ca^{2+} concentrations on promoting pyrophosphatase activity in NH_4OAc-leached Clarion soil at increasing concentrations of PPi; ●, 50 mM PPi; ○, 75 mM PPi; ▲, 100 mM PPi; △, 150 mM PPi

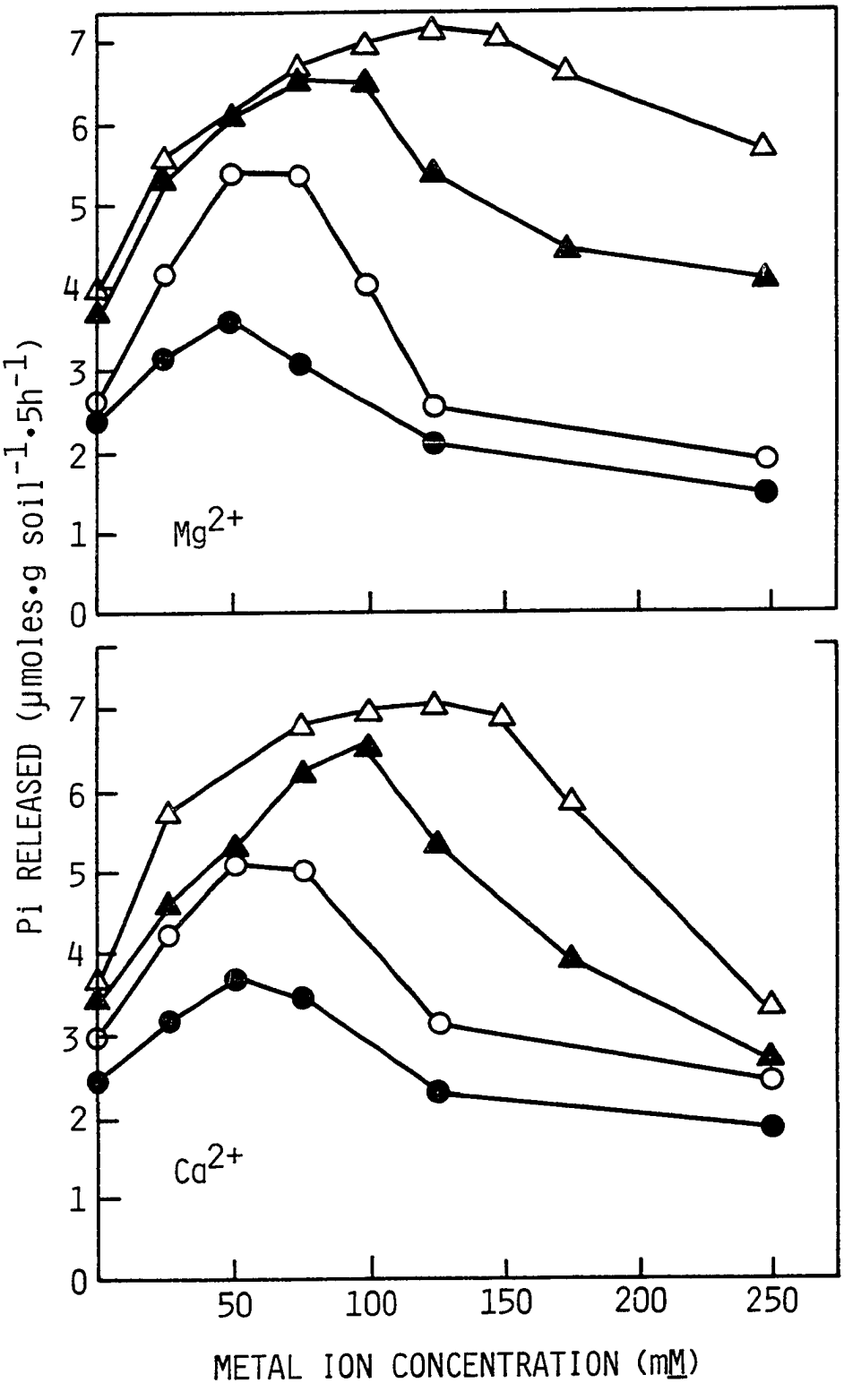
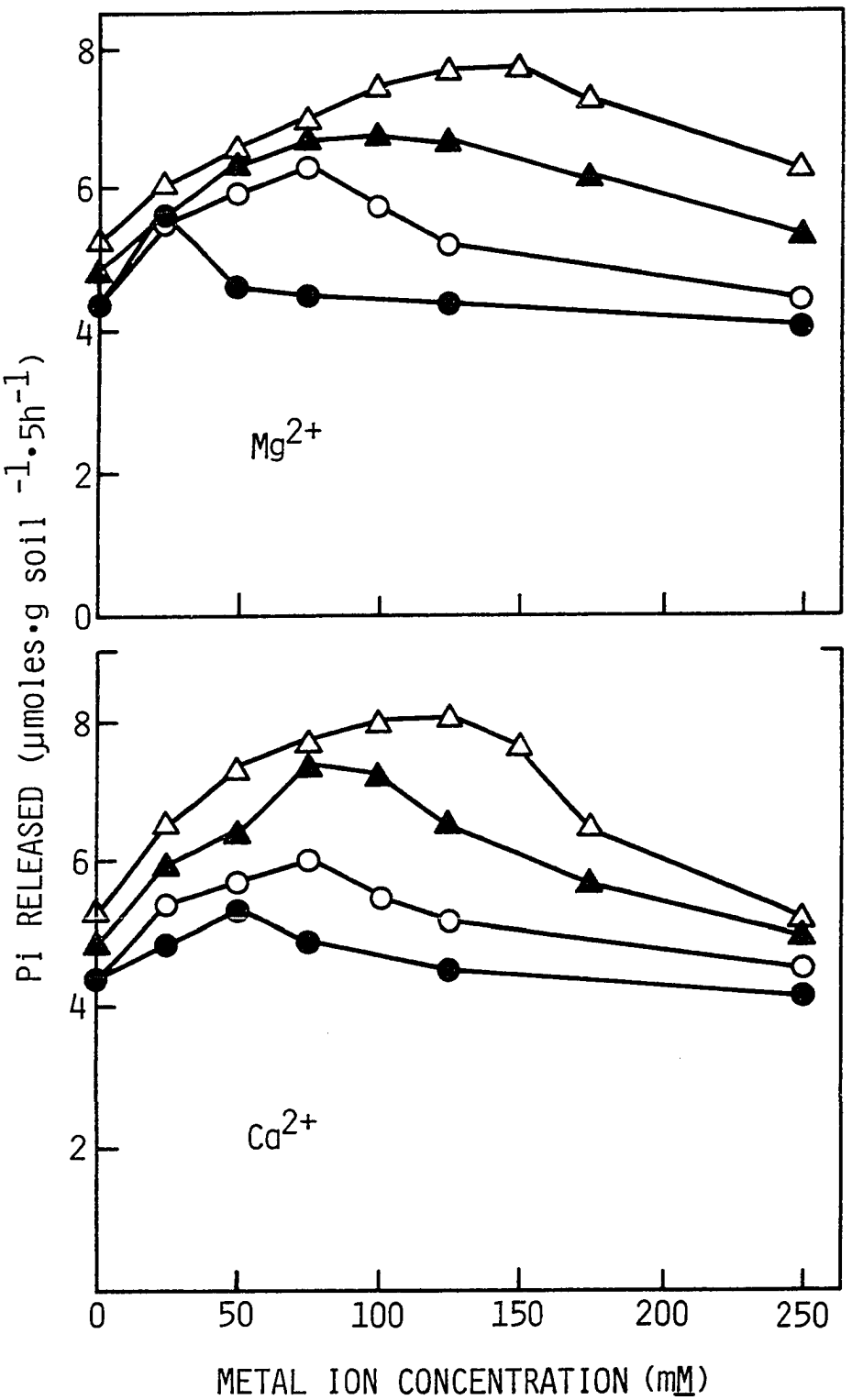

Pi RELEASED (µmoles•g soil^{-1}•5h^{-1})
0
1
2
3
4
5
6
7
Mg^{2+}
Ca^{2+}
50
100
150
200
250
METAL ION CONCENTRATION (mM)

Figure 5. Effects of Mg^{2+} and Ca^{2+} concentrations on promoting pyrophosphatase activity in NH_4OAc-leached Nicollet soil at increasing concentrations of PPi; ●, 50 mM PPi; ○, 75 mM PPi; ▲, 100 mM PPi; △, 150 mM PPi


8
6
4
2
0
Mg^{2+}
8
6
4
2
Ca^{2+}
Pi RELEASED (μmoles•g soil $^{-1}$•5h^{-1})
0
50
100
150
200
250
METAL ION CONCENTRATION (mM)

The highest PPi concentration tested was 150 mM because above this concentration the PPi would not go into solution in the universal buffer at pH 8.

Previous work showed that when soil pyrophosphatase was assayed at a PPi concentration above 60 mM, the enzyme reaction velocity decreased. The magnitude of this decrease was greater as the PPi concentration increased (Dick and Tabatabai, 1978). This lower rate of PPi hydrolysis at high concentrations of PPi could be due to lack of sufficient amount of Mg^{2+} and Ca^{2+} needed to activate PPi (Butler, 1971). The results reported in Figures 4 and 5 support this hypothesis, and show that Mg^{2+} and Ca^{2+} produce the active substrate. At high concentrations, however, above 1:1 Mg^{2+} or Ca^{2+}:PPi, Mg^{2+} and Ca^{2+} inhibited pyrophosphatase activity in the two soils studied. The optimum molar ratio of Mg^{2+}:PPi and Ca^{2+}:PPi was 1:1 in Clarion and Nicollet soils (Table 3). This ratio is lower than that (2:1) reported by Unemoto et al. (1973) for E. coli and by Searle and Hughes (1977) for microorganisms isolated from soil.

To study the degree of protection of pyrophosphatase by Mg^{2+} and Ca^{2+} against heat inactivation, information on the effect of temperature on the activity of this enzyme was needed. For this work samples of three soils (Webster, Clarion, and Okoboji) were exposed to different temperatures for 1 h, then they were brought to room temperature and assayed for pyrophosphatase activity at 37 C. Figure 6 shows that

Table 3. Effect of concentrations of metal ions and pyrophosphate on ratios of Mg^{2+}:PPi and Ca^{2+}:PPi at optimum pyrophosphatase activity in NH_4OAc-leached soils[a]

Soil	Metal ion	PPi conc. (mM)	Optimum metal ion conc. (mM)	Ratio of metal ion:PPi at optimum activity
Clarion	Mg^{2+}	50	50	1:1
		75	50-75	0.67:1-1:1
		100	75-100	0.75:1-1:1
		150	125-150	0.83:1-1:1
	Ca^{2+}	50	50	1:1
		75	50-75	0.67:1-1:1
		100	100	1:1
		150	125-150	0.83:1-1:1
Nicollet	Mg^{2+}	50	25	0.5:1
		75	75	1:1
		100	100	1:1
		150	150	1:1
	Ca^{2+}	50	50	1:1
		75	75	1:1
		100	75-100	0.75:1-1:1
		150	125-150	0.83:1-1:1

[a]Calculated from the results shown in Figures 4 and 5.

pyrophosphatase was stable up to 60 C in both field-moist and air-dried soils. Although the air-dried soils contained much less pyrophosphatase activity at temperatures above 60 C, the rate of inactivation of this enzyme was much faster in the field-moist than in the air-dried soils. Pyrophosphatase activity was completely destroyed in the field-moist soils at 120 C, but the air-dried soils contained some residual

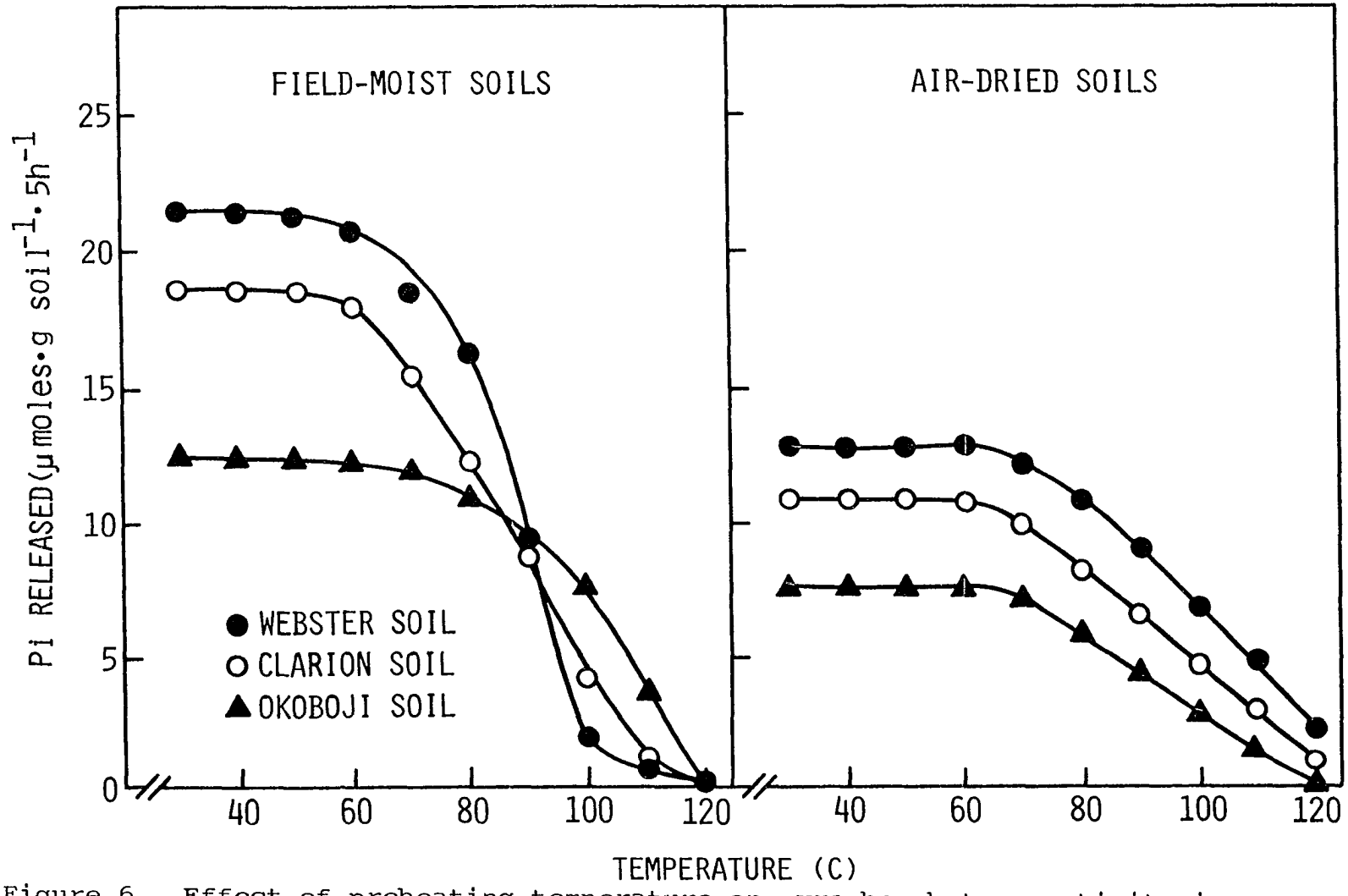
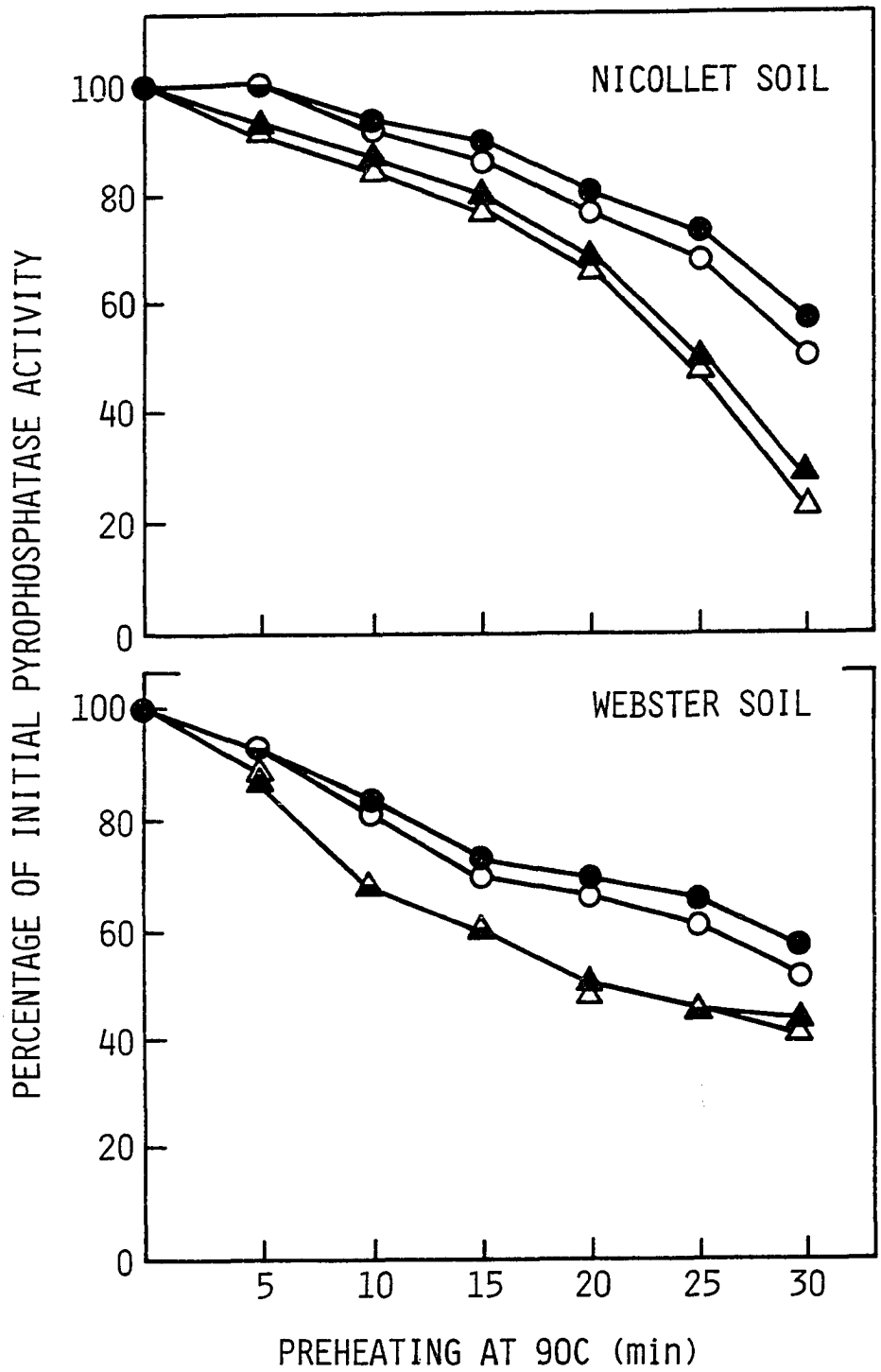


Figure 6. Effect of preheating temperature on pyrophosphatase activity in field-moist and air-dried soils

activity at this temperature.

Figure 7 shows the effect of Ca^{2+} and Mg^{2+} on protection of pyrophosphatase against heat inactivation. In this study, 1 g samples of the NH_4OAc-leached soils were preheated at 90 C for various times before and after treatment with 0.5 ml of 200 m$\underline{M}$ $CaCl_2$ or $MgCl_2$. These results suggest that metal ions such as Ca^{2+} and Mg^{2+} have a function beyond that of binding with PPi to form the active substrate molecule. These metal ions seem to bind to the pyrophosphatase, stabilizing it in its active three-dimensional form. When Ca^{2+} or Mg^{2+} is not present, heat treatment causes greater thermal inactivation than in the presence of these metal ions. Preliminary tests showed that heating 1 g Webster soil at 80 C for 30 min in the presence of 0.5 ml 200 m$\underline{M}$ $CaCl_2$ gave complete protection, with no decrease in activity observed in the subsequent assay. Under similar conditions, but with $MgCl_2$, the enzyme retained 91% of its original activity, but when no metal ion was present only 80% of the original activity was retained. This protection effect of Ca^{2+} and Mg^{2+} was more evident in Nicollet soil than in Webster soil when 1 g samples of these soils were heated at 90 C in the presence of these metal ions. The amount of residual activity in Nicollet soil that did not contain any metal ions when heated for 30 min at 90 C was approximately 30%. However, in the presence of Mg^{2+} the residual activity was 50% and in the presence of Ca^{2+} it was 57% of the initial activity. For Webster soil, 57% of the

Figure 7. Effect of Ca^{2+} and Mg^{2+} on heat inactivation of soil pyrophosphatase; ●, soil treated with 0.5 ml of 200 mM $CaCl_2$ and ○, with 0.5 ml of 200 mM $MgCl_2$ before heat treatment; in both treatments the soil samples received 0.5 ml of water after heating (before pyrophosphatase assay); the triangles are control soil samples treated with 0.5 ml of water before heat treatment, but ▲ indicates soil received 0.5 ml of 200 mM $CaCl_2$ after heating and △ indicates soil received 0.5 ml of 200 mM $MgCl_2$ after heating (before pyrophosphatase assay)


NICOLLET SOIL
WEBSTER SOIL
PERCENTAGE OF INITIAL PYROPHOSPHATASE ACTIVITY
PREHEATING AT 90C (min)
0
20
40
60
80
100
5
10
15
20
25
30

initial activity remained after heating at 90 C for 30 min in the presence of Ca^{2+}, 51% of the initial activity remained when Mg^{2+} was present, but only 42% when no metal ions were present during the preheating treatment. These results suggest that Ca^{2+} and Mg^{2+} may aid in stabilizing pyrophosphatase in the soil environment.

Dick and Tabatabai (1978) reported that Na_2CO_3 strongly inhibits soil pyrophosphatase activity. Free carbonates (primarily as $CaCO_3$) in soils also seem to influence pyrophosphatase. This pyrophosphatase activity is strongly and negatively correlated ($r = -0.97^{**}$) with the percentage $CaCO_3$ equivalent in soils (Dick and Tabatabai, 1979). Therefore, the effect of Mg^{2+} and Ca^{2+} on the pyrophosphatase activity in the NH_4OAc-leached Webster and Nicollet soils treated with Na_2CO_3 was studied. Assay of pyrophosphatase in the NH_4OAc-leached soils in the presence of 5 m$\underline{M}$ Na_2CO_3 resulted in activity 91 and 98% of the initial pyrophosphatase activity in Nicollet and Webster soil, respectively, not treated with Na_2CO_3 (Table 4). The inhibition of this enzyme by Na_2CO_3 could be reversed by the addition of Mg^{2+} and Ca^{2+}. The pyrophosphatase activities in the presence of 5 m$\underline{M}$ Na_2CO_3 and at optimum Mg^{2+} and Ca^{2+} concentrations resulted in activity of this enzyme of 103 and 117%, respectively, when compared to the untreated Webster soil. The pyrophosphatase activity in the Nicollet and Webster soils, in the presence of 20 m$\underline{M}$ $NaCO_3$, however, resulted in activity 58 and 66%, respectively,

Table 4. Pyrophosphatase activity in NH_4OAc-leached soils after treatment with sodium carbonate and magnesium or calcium ions

Soil	Metal ion	Metal ion conc. (mM)	Sodium carbonate concentration (mM) 0	5	20
			---Pyrophosphatase activity[a]--		
Webster	Mg^{2+}	0	6.4	6.3 (98)	4.2 (66)
		20		6.6 (103)	5.5 (86)
		35		6.4 (100)	5.0 (78)
		50		6.2 (97)	3.7 (58)
		70		5.0 (78)	3.3 (52)
		100		2.8 (44)	3.0 (47)
	Ca^{2+}	0	6.4	6.3 (98)	4.2 (66)
		20		7.5 (117)	4.8 (75)
		35		7.4 (116)	4.8 (75)
		50		6.4 (100)	3.4 (53)
		70		5.7 (89)	2.7 (42)
		100		3.2 (50)	2.5 (39)
Nicollet	Mg^{2+}	0	4.3	3.9 (91)	2.5 (58)
		20		4.8 (112)	2.8 (65)
		35		4.9 (114)	3.2 (74)
		50		3.9 (91)	2.3 (53)
		70		3.1 (72)	2.0 (47)
		100		2.2 (51)	1.9 (44)
	Ca^{2+}	0	4.3	3.9 (91)	2.5 (58)
		20		5.1 (119)	3.5 (81)
		35		5.9 (137)	3.6 (84)
		50		5.3 (123)	3.8 (88)
		70		4.3 (100)	3.2 (74)
		100		2.2 (51)	1.9 (44)

[a] μmoles Pi released·g $soil^{-1}$·5 h^{-1}. Figures in parentheses are the pyrophosphatase activities expressed as a percentage of the initial activity in the leached soil, not treated with sodium carbonate.

of that initially determined for these soils not treated with $NaCO_3$ (Table 4). This inhibition by Na_2CO_3 was not due to change in pH because upon addition of Na_2CO_3 to the assay mixture, less than a 0.2 pH unit change occurred. Addition of Mg^{2+} and Ca^{2+} could reverse a portion of the inhibition of pyrophosphatase caused by Na_2CO_3. The activity of this enzyme when treated with 20 m$\underline{M}$ Na_2CO_3 and at optimum Mg^{2+} and Ca^{2+} concentrations was 86 and 75%, respectively, of the initial activity in Webster soil not treated with Na_2CO_3, and 74 and 88%, respectively, of the initial activity in Nicollet soil not treated with Na_2CO_3 (Table 4).

The decrease in pyrophosphatase activity observed on addition of Na_2CO_3 was initially considered to be due to precipitation of residual Mg^{2+} and Ca^{2+} not removed by the NH_4OAc-leaching procedure. Therefore, the addition of Mg^{2+} and Ca^{2+} to bind the carbonate and remove its inhibitory effect should cause complete recovery of activity of this enzyme. However, this was not observed when the NH_4OAc-leached soils were treated with 20 m$\underline{M}$ Na_2CO_3 and sufficient Mg^{2+} and Ca^{2+} added to theoretically bind all the added carbonate. The effect of carbonates on soil pyrophosphatase activity remains to be investigated.

PART II. HYDROLYSIS OF ORGANIC AND INORGANIC PHOSPHORUS COMPOUNDS ADDED TO SOILS

INTRODUCTION

Use of inorganic phosphate compounds as fertilizer sources of P for plant growth and development is a common agricultural practice. The reactions of inorganic phosphate in soils have been studied extensively, and the accumulated information indicates that inorganic phosphate moves very little, if any, in soils (for review of literature, see Hemwall, 1957). The mechanisms that restrict the movement of orthophosphate in soils include sorption by soil constituents and precipitation by multivalents cations (Taylor and Gurney, 1965a,b).

One of the proposed solutions to the problem of P fixation in soils is use of organic phosphate or inorganic P compounds that remain soluble in water. Such P compounds are not readily fixed in soils and may move away from the site of application. Pinck et al. (1941) studied the rate of change in concentrations of several water-soluble organic P compounds to less water-soluble forms in soils. They reported that some organic phosphates such as potassium diphenyl phosphate, potassium diphenyl pyrophosphate, and calcium diethyl phosphate tended to remain water soluble for several days and were considered to have some advantages over inorganic phosphates because of their greater penetrability. Other organic P compounds studied, such as the un-ionized triethyl and trimethyl phosphates, were completely recovered

from two of the three soils even after 3 weeks of incubation under aerobic conditions at 25 C. Rolston et al. (1974) studied glycerophosphate as a potential source of organic phosphate fertilizer and concluded that it moved greater distances into a column of a calcareous soil than did inorganic phosphate (potassium phosphate). Rolston et al. (1975) evaluated the relative movement of glycerophosphate, methyl ester phosphate, ethyl ester phosphate, glycol phosphate, glucose-1-phosphate, and glucose-6-phosphate in soils and reported that these compounds moved at similar rates in soils. The similarity in movement of these P compounds in soils was supported by experiments showing that all these compounds were hydrolyzed at similar rates by acid phosphatase (source not specified). The relative rates of hydrolysis of some of these P compounds in soils remains to be investigated.

Studies of hydrolysis of organic and inorganic P compounds in soils have been hampered by lack of methods for determination of orthophosphate in the presence of acid-labile P compounds. Several of the P compounds recommended as soil amendments (e.g., glucose-1-phosphate) are acid labile and hydrolyze in the acid media used for colorimetric determination of orthophosphate by the heteropoly blue method. Recently, we developed a colorimetric method for determination of orthophosphate in aqueous solutions containing labile P compounds (Dick and Tabatabai, 1977a). This method involves a rapid formation of heteropoly blue in the presence of

ascorbic acid-trichloroacetic acid and citrate-arsenite reagents and complexation of the excess molybdate ions to prevent further formation of blue color from the orthophosphate derived from hydrolysis of the acid-labile P compounds. We used this method in the work reported (1) to study the rates of hydrolysis of seven organic phosphates and two inorganic P compounds in soils incubated under aerobic and waterlogged conditions, and (2) to determine the effects of the degree of substitution of organic moieties for the hydrogens of orthophosphoric acid on the rate of hydrolysis.

DESCRIPTION OF METHODS

The soils used (Table 5) were from surface soils (0-15 cm) selected to give a range in pH, organic matter content, organic P, inorganic P, and texture. Before use, each sample was air-dried and crushed to pass a 2-mm screen. A subsample of each soil was ground to pass an 80-mesh sieve. In the analyses reported in Table 5, pH was determined by a glass electrode (soil:water ratio, 1:2.5), organic C by the method of Mebius (1960), total P by the method of Dick and Tabatabai (1977b), and inorganic P as described by Olsen and Dean (1965). Organic P was determined by subtracting the value of inorganic P from the value obtained for total P. The particle-size distribution was determined by the pipette analysis of Kilmer and Alexander (1949). Organic C, total P, and inorganic P were performed on the <80-mesh samples. All other analyses were performed on the coarser, <2-mm, soil samples.

Before use, each P compound (Table 6) was analyzed for total P. In this analysis, 0.20 g of the P compound in a 25-ml volumetric flask was treated with 3 ml of conc. HCl and boiled for 15 min on a sand bath adjusted at 200 C. After cooling to room temperature, the volume was adjusted with deionized water and mixed, and an aliquot was taken for orthophosphate analysis by the method of Dick and Tabatabai (1977a). The phosphomonoesterase and phosphodiesterase activity values reported in Table 7 are those obtained by the

Table 5. Properties of soils used

Soil type	Soil classification	pH	Organic C (%)	Phosphorus (ppm)		Clay (%)	Sand (%)
				Organic	Inorganic		
Clarion 1	Typic Hapludoll	4.6	1.99	304	236	24	37
Nicollet cl	Aquic Hapludoll	6.2	2.73	284	186	29	34
Harps cl	Typic Calciaquoll	7.6	3.24	392	272	30	31

methods of Eivazi and Tabatabai (1977) and Browman and Tabatabai (1978), respectively.

The rates of hydrolysis of the P compounds in soils were determined under two conditions, aerobic and waterlogged. To determine the rate of hydrolysis under aerobic conditions, 2 g soil in a 50-ml plastic centrifuge tube were treated with 1 ml solution (70% of water-holding capacity) containing 1 mg P of the compound specified (500 ppm P on soil basis). This solution was added dropwise to moisten the whole soil sample. The tube was stoppered and incubated at 20 C for 1, 2, 4, or 7 days. The tube was aerated daily by removing the stopper and flushing the tube with air. The moisture was adjusted by weighing the tube and adding deionized water to compensate for the moisture lost. The incubation under waterlogged conditions was similar to that under aerobic conditions, but the 1 mg P of the compound studied was added in 5 ml solution, and the tube was not aerated during incubation. After incubation, 1 ml of water was added in the case of aerobic or 5 ml of water in the case of waterlogged conditions, and the orthophosphate produced from each compound was extracted with 25 ml $\underline{N}$ H_2SO_4 and determined as described by Dick and Tabatabai (1978).

Controls were performed to correct for orthophosphate in samples, but not derived from hydrolysis of the P compounds, and for any trace amount of orthophosphate that may be present in these compounds. For controls, the 2-g samples were treated

with 1 ml or 5 ml water and incubated along with the P-treated soils. After incubation, 1 ml or 5 ml solution containing 1 mg P of the corresponding compound was added to the controls incubated under aerobic and waterlogged conditions, respectively, and the orthophosphate was extracted and determined as described for the P-treated samples. The standard error of estimating the orthophosphate produced from the P compounds studied is <2 μg P (Dick and Tabatabai, 1978).

All values reported are averages of duplicate determinations expressed on moisture-free soil, moisture being determined from loss in weight after drying at 105 C for 24 hours.

RESULTS AND DISCUSSION

Table 6 shows the formulae and chemical composition of the organic and inorganic P compounds used in this study. The percentage P content of the compounds used ranged from about 8% in p-nitrophenyl phosphate, bis-p-nitrophenyl phosphate, and glucose-1-phosphate to 37% in phosphonitrilic hexaamide. In addition to their content of P, monomethyl phosphate, phosphonitrilic hexaamide, and ammonium tetrametaphosphimate also contained 54.2%, 50.6%, and 24.6% of N, respectively.

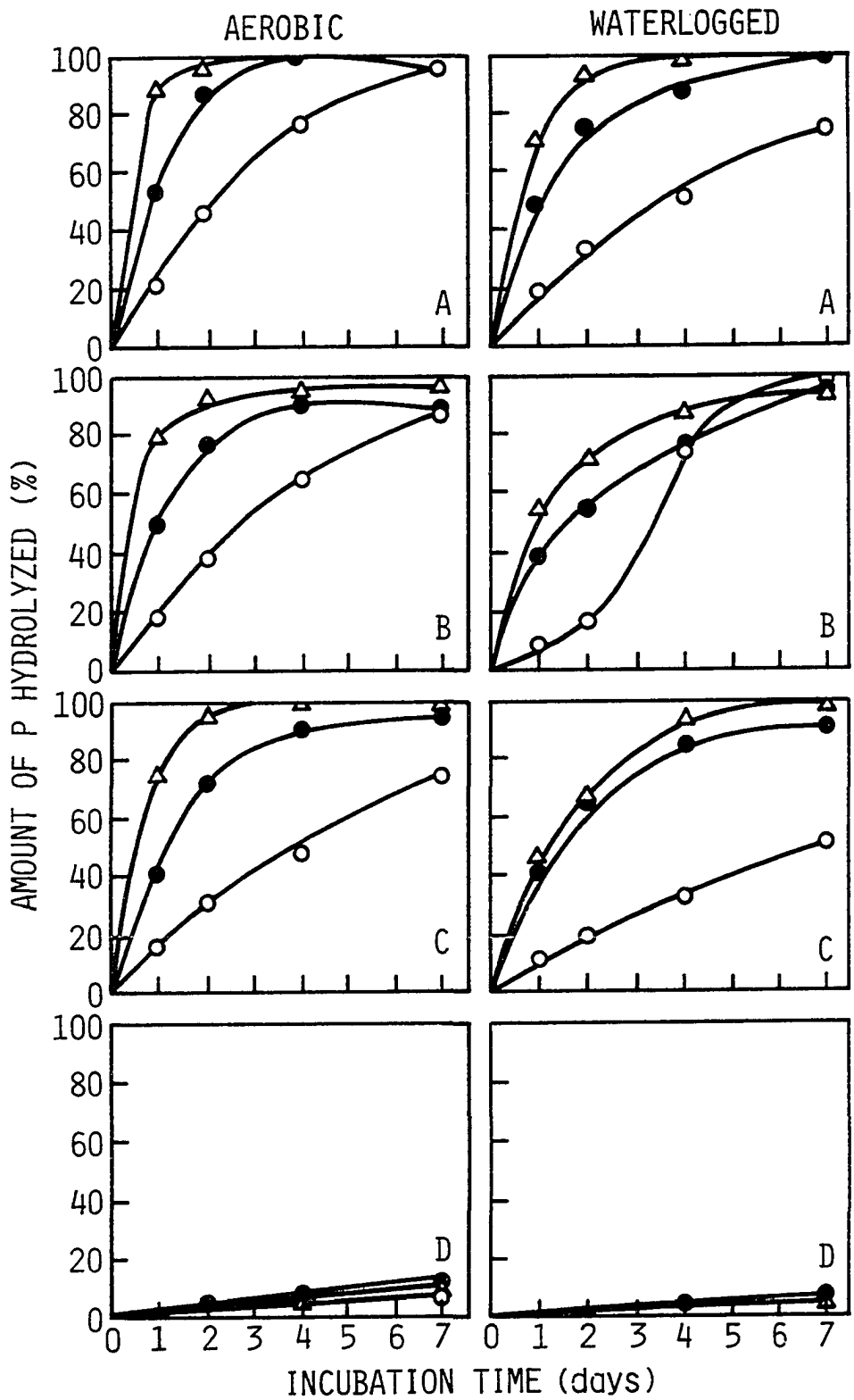
The rates of hydrolysis of the P compounds in soils were studied at 20 C because this temperature approximates the mean of soil temperatures in temperate regions during the spring and summer. Therefore, the observed rate of hydrolysis should give a reliable estimate of the comparative behavior of these compounds under field conditions. The rates of hydrolysis of soil organic P compounds are known to be strongly influenced by temperature; increase in rate of hydrolysis is observed with increasing temperature, especially above 30 C (Thompson and Black, 1947; van Diest and Black, 1959). The concentration of P added to the soils (500 ppm P on soil basis) was designed to approximate that which occurs near a fertilizer band.

The effects of time and conditions of incubation on the amount of P hydrolyzed from the P compounds studied in the three soils are shown in Figures 8 and 9. The rates of

Table 6. Properties of phosphorus compounds used

P compound			P content (%)		N content (%)
Name	Formula	Molecular weight	Theoretical	Determined	
Monomethyl phosphate	$[(NH_2)_6H]_2CH_3PO_4 \cdot 1/3H_2O$	310	10.0	9.7	54.2
Phenyl phosphate	$Na_2C_6H_5PO_4$	218	14.2	14.2	0
Diphenyl phosphate	$(C_6H_5O)_2P(O)OH$	250	12.4	12.2	0
p-nitrophenyl phosphate	$Na_2C_6H_4NO_2PO_4 \cdot 6H_2O$	371	8.3	8.2	0
bis-p-nitrophenyl phosphate	$Na(C_6H_4NO_2)_2PO_4$	362	8.5	8.5	0
β-glycerophosphate	$Na_2C_3H_7O_2PO_4 \cdot 3H_2O$	270	11.5	11.3	0
α-D-glucose-1-phosphate	$Na_2C_6H_8O_5PO_4 \cdot 3.5H_2O$	364	8.5	8.5	0
Phosphonitrilic hexaamide	$P_3N_3(NH_2)_6 \cdot H_2O$	249	37.3	37.2	50.6
Ammonium tetrametaphosphimate	$(NH_4)_4(PO_2NH)_4 \cdot 4H_2O$	456	27.2	26.9	24.6

Figure 8. Effect of incubation time on hydrolysis in soils of monomethyl phosphate (A), β-glycerophosphate (B), α-D-glucose-1-phosphate (C), and phosphonitrilic hexaamide (D) under aerobic and waterlogged conditions; △, Nicollet soil; ●, Clarion soil; ○, Harps soil


AEROBIC
WATERLOGGED
AMOUNT OF P HYDROLYZED (%)
INCUBATION TIME (days)
A
B
C
D
0
20
40
60
80
100
0 1 2 3 4 5 6 7

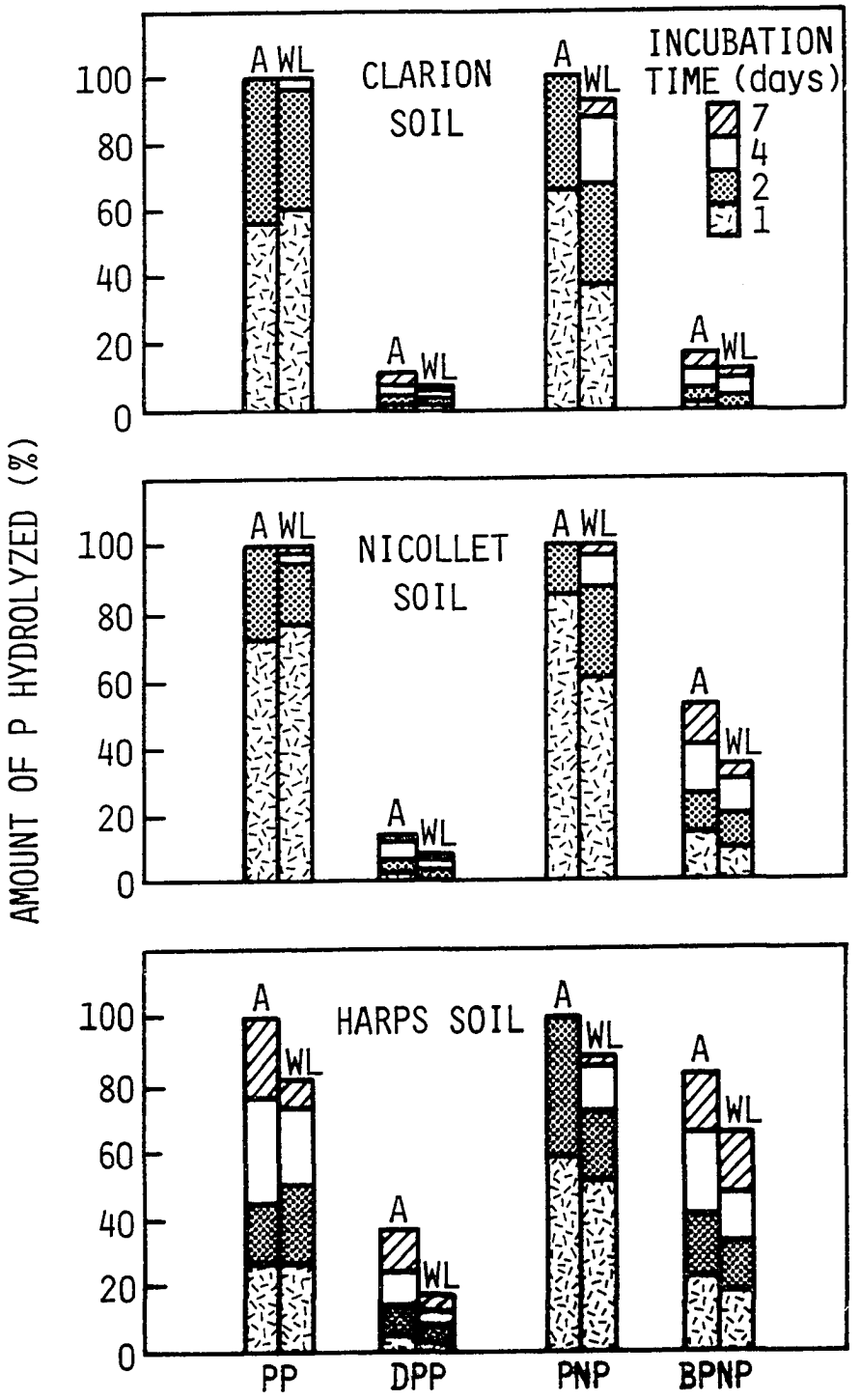


Figure 9. Effect of incubation time on hydrolysis in soils of phenyl- (PP), diphenyl- (DPP), p-nitrophenyl- (PNP), and bis-p-nitrophenyl (BPNP) phosphate under aerobic (A) and waterlogged (WL) conditions

hydrolysis of the P compounds tested varied considerably. The highest rates occurred with the monosubstituted orthophosphoric acid, monomethyl phosphate, β-glycerophosphate, glucose-1-phosphate, phenyl phosphate, and p-nitrophenyl phosphate, all of which were completely hydrolyzed in the acid soils (Clarion and Nicollet) after 7 days of incubation. The other P compounds tested were not completely hydrolyzed in one week under either aerobic or waterlogged conditions. The average percentage of hydrolysis of diphenyl phosphate, bis-p-nitrophenyl phosphate, and phosphonitrilic hexaamide hydrolyzed in the three soils during 7 days of incubation under aerobic conditions were 21, 51, and 10%, respectively. The corresponding averages of percentage of these P compounds hydrolyzed in the soils under waterlogged conditions were 11, 37, and 3%. Ammonium tetrametaphosphimate was not hydrolyzed during the 7 days of incubation under aerobic or anaerobic conditions.

Rolston et al. (1975) reported that β-glycerophosphate, monomethyl phosphate, and glucose-1-phosphate infiltrated into soils at similar rates and showed that these P compounds were hydrolyzed at similar rates by an acid phosphatase. We found that the rates of hydrolysis of these compounds in each soil were similar, but the rates varied among the three soils studied (Figure 8). The rates of hydrolysis of β-glycerophosphate, monomethyl phosphate, and glucose-1-phosphate were higher in the acid Clarion and Nicollet soils than in the calcareous Harps soil. Hydrolysis in soils of P in ester linkages

generally is considered due to the catalytic effect of phosphatases. Table 7 shows the activity of phosphomonoesterases (acid and alkaline phosphatases) and phosphodiesterase in the soils used. Clarion and Nicollet soils contained higher acid phosphatase activity than did the Harps soil. The distribution of acid and alkaline phosphatases in the soils used supports our previous finding that acid phosphatase is predominant in acid soils and that alkaline phosphatase is predominant in alkaline soils (Eivazi and Tabatabai, 1977; Juma and Tabatabai, 1978). Because alkaline phosphatase activity is observed mainly at high pH values (optimum activity is observed with tris buffer pH 11), acid phosphatase activity seems the main determining factor in hydrolysis of organic P compounds added to soils. This conclusion is supported by the results shown in Figures 8 and 9 and Table 7 and those reported earlier by Pinck et al. (1941) and Rolston et al. (1975) on greater movement of organic P compounds in calcareous soils than in acid soils. The high acid phosphatase activity in acid soils causes a rapid hydrolysis of organic P to orthophosphate, which is fixed.

Figure 9 shows the effect of degree of hydrogen substitution by organic moeities in orthophosphoric acid on the rates of hydrolysis of the P compounds. The rates of hydrolysis of diphenyl phosphate and bis-p-nitrophenyl phosphate in the three soils studied were considerably lower than those of their monosubstituted counterparts, phenyl phosphate and

Table 7. Phosphatase activity of the soils used

Soil	Phosphomonoesterase activity[a]		Phosphodiesterase activity[a]
	Acid phosphatase	Alkaline phosphatase	
Clarion	130 (70)[b]	24	16 (5)[b]
Nicollet	195 (165)	53	67 (42)
Harps	78 (68)	230	113 (105)

[a] μg p-nitrophenol released·g soil^{-1}·h^{-1}.

[b] Figures in parentheses indicate phosphatase activity in the absence of buffer.

p-nitrophenyl phosphate, especially in the acid Clarion soil. The reason for this lower rate of hydrolysis of diphenyl phosphate and bis-p-nitrophenyl phosphate is that both compounds require twofold hydrolysis to change their constituent P to orthophosphate. The observed highest rate of hydrolysis of the disubstituted orthophosphoric compounds in Harps soil compared with those in the other two soils seems due to highest phosphodiesterase activity in the Harps soil (Table 7).

Generally, the rates of hydrolysis of P in the compounds tested were higher under aerobic than under waterlogged conditions. This difference in rates of hydrolysis is partly due to the higher concentration of P in the soil solution under aerobic than under waterlogged conditions. Another factor that might have contributed to the lower rate of hydrolysis

in waterlogged soils is the orthophosphate produced from the increased solubility of Fe and Al phosphate under these conditions (Patrick and Fontenot, 1976). Orthophosphate has been shown to be a competitive inhibitor of phosphatases in soils (Browman and Tabatabai, 1978; Juma and Tabatabai, 1978).

Among the compounds containing N (Table 6), the rate of hydrolysis of monomethyl phosphate (cyclohexane ammonium salt) was the most rapid; 100% of the P added was released during 7 days of incubation under aerobic conditions. The inorganic P compounds tested, phosphonitrilic hexaamide and ammonium tetrametaphosphimate, were hydrolyzed at an extremely slow rate; no apparent hydrolysis was evident from ammonium tetrametaphosphimate after 7 days of incubation at 20 C under aerobic conditions. The amount of phosphonitrilic hexaamide hydrolyzed in samples of the three soils studied ranged from 7% in Harps soil to 13% in Clarion soil. The availability of P in these compounds to plants remains to be investigated.

PART III. PHOSPHATASES IN PLANT MATERIALS, MANURES, SEWAGE SLUDGES, AND SOILS

INTRODUCTION

Phosphatases are widely distributed hydrolytic enzymes that catalyze the hydrolysis of both esters and anhydrides of phosphoric acid (Schmidt and Laskowski, 1961). The detection of phosphatase activity in soils by Rogers (1942) has stimulated much interest in their role in the soil environment. This interest in phosphatases in soils is largely derived from the importance of P in plant nutrition. The Commission on Enzymes of the International Union of Biochemistry has classified the phosphatases into five major groups (Florkin and Stotz, 1964). These include the phosphoric monoester hydrolases (EC 3.1.3), phosphoric diester hydrolases (EC 3.1.4), triphosphoric monoester hydrolases (EC 3.1.5), enzymes acting on phosphoryl-containing anhydrides (EC 3.6.1), and enzymes acting on P-N bonds (EC 3.9), such as the phosphoamidases (EC 3.9.1.1). Most of the investigations of phosphatases in soils have been concerned with the activity of phosphomonoesterases, especially acid phosphatase. However, in addition to phosphomonoesterases, pyrophosphatase, metaphosphatase, and phytase have been detected in soils (Skujins, 1967). Recently, Eivazi and Tabatabai (1977) detected phosphodiesterase and phosphotriesterase activity in soils, that were capable of hydrolyzing bis-p-nitrophenyl phosphate and tris-p-nitrophenyl phosphate, respectively.

The source of phosphatases in soils are microorganisms,

plant residues, and plant roots (Skujins, 1976). Pure cultures of many microorganisms commonly found in soils have been shown to produce exocellular phosphatases (Casida, 1959; Greaves et al., 1963; Cosgrove et al., 1970; Ko and Hora, 1970). The presence of plants and the type of plants grown in a soil also have a marked effect on soil phosphatase activity (Neal, 1973; Khan, 1970; Beck, 1974). The changes in phosphatase activity are related to changes in soil organic matter content and microbial populations brought about by plants. In addition, phosphatase activity in soils is greatly influenced by plant root rhizospheres with rhizosphere phosphatase activities generally higher than are activities remote from the root (Spier and Ross, 1978). The increased acid phosphatase activity associated with the rhizosphere of wheat plant in a South Australian soil showed that activity was located at the plasmalemma and in the cell walls of soil bacteria, and in the outer corticle cells of wheat root. Activity was also associated with fragments of finely divided plant cell wall materials (see Ladd, 1978).

Besides plant materials, other materials containing phosphatases may enter the soil environment. Disposal of manures on soils to improve soil fertility is a common agricultural practice. In addition, land application of sewage sludges is becoming more prevalent. To date, little information has been obtained to assess the contribution of such materials to soil enzyme activity.

This study was undertaken to compare the phosphatases in plant materials, manures, sewage sludges, and soils. The phosphatases studied were acid and alkaline phosphatases, phosphodiesterase, and inorganic pyrophosphatase. Comparing the phosphatases in the organic waste materials and soils was done by comparing important kinetic parameters of enzymes such as pH optima, Michaelis constants (K_m), maximum rates of reaction (V_{max}), and energies of activation (E_a). Furthermore, acid phosphatase and inorganic pyrophosphatase activity in soils were measured during the course of a 4-month incubation period, after being treated with plant material, manure, and sewage sludge to study the long-term effect of such materials on these enzymes in soils.

DESCRIPTION OF METHODS

The materials used in this study (Table 8) were prepared in the following manner. The soils were collected to give a range in pH values and organic C content and were air-dried and crushed to pass a 2-mm screen. The plant materials were harvested from the field in early July, air-dried for 3 days, and ground to pass a 20-mesh sieve. The sewage sludge samples were collected from wastewater treatment plants in Iowa. The samples were liquid sewage sludge collected in plastic containers, brought to the laboratory, the pH of the samples measured, and then air-dried. To speed up the air-drying process, the sludge samples were spread on a tray 36 x 39 cm lined with aluminum foil and placed in a well-ventilated hood. A fan was directed toward the hood to enhance the drying process. After the sewage sludge samples were dried, they were ground to pass a 60-mesh sieve. The manure samples used in this study were collected fresh and dried in a similar manner to that described above for the sewage sludge samples. After the manure samples were dried, they were ground to pass a 20-mesh sieve. The plant materials, sewage sludges, and manures prepared were stored in glass bottles at 4 C.

In the chemical analyses reported in Table 8, pH was determined by a glass electrode, organic C by the method of Mebius (1960), total N by the semimicro-Kjeldahl digestion

Table 8. Chemical properties of soils and waste materials used

Material	pH[a]	Organic C	Total N	Total P
		----------%----------		
Soils				
Clarion	4.6	1.99	0.17	0.056
Webster	6.5	2.91	0.25	0.054
Okoboji	7.0	5.32	0.42	0.079
Canisteo	7.7	3.11	0.28	0.074
Plant materials				
Oat	5.9	43.7	1.83	0.27
Alfalfa	5.9	43.7	2.09	0.23
Corn	6.1	43.1	2.34	0.35
Soybean	6.8	38.4	4.12	0.38
Manures				
Cow	5.9	44.6	2.21	0.40
Hog	6.2	45.6	2.87	1.20
Chicken	7.7	38.0	2.28	0.90
Horse	8.1	46.9	1.74	0.39
Sewage sludges[b]				
Shellsburg (IT)	5.3	46.7	2.58	0.90
Boone (RP)	6.1	34.7	2.47	0.61
Des Moines (PD)	6.6	32.8	2.81	1.17
Ames (SD)	7.0	33.9	1.52	1.36

[a]Soil pH values were measured in deionized water at a soil:water ratio of 1:2.5, plant material and manure pH values were measured in deionized water at a plant material:water or manure:water ratio of 1:10, and sewage sludge pH values were measured before drying the samples.

[b]The sources of sewage sludge were IT, Imhoff tank; RP, raw primary; PD, primary digester; and SD, secondary digester.

method described by Bremner (1960), and total P by the NaOBr-oxidation procedure developed by Dick and Tabatabai (1977b). For the sewage sludges, total N was determined by the method of Nelson and Sommers (1972) and total P by digesting 0.4 g sludge material with 2 ml HNO_3 and 2 ml $HClO_4$ in a 30-ml Kjeldahl flask and then removing the excess HNO_3 by boiling the sample with 3 ml HCl. The sludge digest thus obtained was diluted to 50 ml volume with water and the phosphorus was determined by the method of Murphy and Riley (1962). A complete chemical analysis of the sewage sludge materials used is reported by Tabatabai and Frankenberger (1979).

Assays for acid and alkaline phosphatase were performed as described by Eivazi and Tabatabai (1977). This method is based upon colorimetric determination of the p-nitrophenol released when material containing phosphatase is incubated for 1 h at 37 C with 0.2 ml toluene, 4 ml universal buffer, and 1 ml of substrate, p-nitrophenyl phosphate, made in the corresponding buffer. The assay procedure for phosphodiesterase was similar to that of assay of the phosphomonoesterases with the exception that 1 ml of bis-p-nitrophenyl phosphate was used as the substrate. Pyrophosphatase was assayed as described by Dick and Tabatabai (1978). This method is based on determination of the amount of Pi released when material containing pyrophosphatase is incubated with tetrasodium pyrophosphate, made in universal buffer, for 1 h at 37 C. The sample sizes used in measuring activity of the

various phosphatases studied varied, depending on the enzyme and material studied. To assay the four phosphatases studied in soils, 1 g sample sizes were used, while the sample sizes used to assay these enzymes in sewage sludges was 100 mg. For assay of the phosphatases in plant materials and manures, sample sizes used ranged from 1 to 50 mg and from 10 to 100 mg, respectively.

The concentration of substrate in the assay mixture used to determine the optimum buffer pH and E_a for each phosphatase was 1 mM for acid and alkaline phosphatases and phosphodiesterase and 50 mM for pyrophosphatase. The K_m, V_{max}, and E_a values were all determined at the pH of optimum activity found for the different materials. To determine the K_m and V_{max} values for acid and alkaline phosphatase, the substrate concentrations in the assay mixture were 1.0, 1.3, 2.0, 4.0, and 20.0 mM. For phosphodiesterase, the substrate concentrations in the assay mixtures were 0.33, 0.40, 0.50, 0.67, and 1.00 mM and for pyrophosphatase, the substrate concentrations were 10.0, 12.5, 16.7, 25.0, and 50.0 mM for determination of the K_m and V_{max} values in the soils and plant materials and 2.0, 2.5, 3.3, 5.0, and 10.0 mM for determination of these values in the manures.

The effect of soils on acid phosphatase and pyrophosphatase activity of corn root homogenate added to soils was also investigated. To prepare the corn root homogenate used in this study, corn seeds were germinated under sterile condi-

tions as described by Juma (1976). After germination, roots which appeared healthy and free of microbial growth were excised below the seed, weighed, and homogenized in universal buffer (pH 4 for subsequent acid phosphatase assay and pH 6 for subsequent pyrophosphatase assay). The corn roots were homogenized by use of a 25-ml tissue grinder (Kontes of Illinois, Franklin Park, IL). The homogenates were immediately frozen and stored at -20 C until use.

To test the effect of soils on the activity of corn root acid phosphatase, 1 g soil was weighed into a 50-ml Erlenmeyer flask. To each sample was added 0.2 ml toluene, 3.0 ml universal buffer (pH 4) and 1 ml of universal buffer containing 20 mg homogenized corn root. The samples were then assayed for acid phosphatase by using 1 ml of 5 m$\underline{M}$ $\underline{p}$-nitrophenyl phosphate as substrate (Eivazi and Tabatabai, 1977). To test the effect of soils on corn root pyrophosphatase, 1 g soil was weighed into a 50-ml plastic centrifuge tube. The samples were treated with 1 ml universal buffer (pH 6) containing 20 mg homogenized corn root, and assayed for pyrophosphatase by using 2 ml universal buffer (pH 6) containing tetrasodium pyrophosphate to give 50 m$\underline{M}$ PPi (Dick and Tabatabai, 1978). Acid phosphatase and pyrophosphatase were also assayed in 20 mg corn root homogenate as described above for these enzymes, but without the addition of 1 g soil to the assay mixture.

The following procedure was used to study the effect of incubating plant material, manure, and sewage sludge on acid

phosphatase and inorganic pyrophosphatase activity in Webster and Canisteo soils. In this study, 1 g soil was weighed into a 50-ml Erlenmeyer flask or a 50-ml plastic centrifuge tube for the acid phosphatase and pyrophosphatase experiments, respectively. The soil samples each received one of the following treatments: (1) control, (2) 3 mg corn plant, (3) 3 mg steam sterilized corn plant, (4) 3 mg corn plant + 0.5 ml 0.05% sodium azide, (5) 3 mg Shellsburg sewage sludge, (6) 3 mg steam sterilized Shellsburg sewage sludge, (7) 3 mg Shellsburg sewage sludge + 0.5 ml 0.05% sodium azide, (8) 3 mg hog manure, (9) 3 mg steam sterilized hog manure, and (10) 3 mg hog manure + 0.5 ml 0.05% sodium azide. Several soil samples were also included during the incubation period to serve as soil blanks in the subsequent acid phosphatase and pyrophosphatase assay procedures. The soils were wetted (except those treated with sodium azide) with 0.5 ml deionized water and incubated at 25 C. After 0, 1, 2, 4, 7, 10, 15, 20, 30, 60, 90, or 120 days of incubation, a set of control, treated samples, and a blank of each soil were removed from the incubator and assayed for phosphatase activity. However, 0.5 ml less of buffer was added to the assay mixture to compensate for the 0.5 ml water already in the sample. During the course of the incubation period, the samples were aerated every 3 days by removing the stoppers for one-half hour. Several samples were randomly selected, weighed, and the amount of water needed to retain the 0.5 ml in the soil

sample determined. This water was then added to all the soil samples still being incubated, the stoppers replaced, and incubation resumed. The pH values (soil:water ratio, 1:2.5) of the soil samples before and after the total incubation period of 120 days were also measured.

RESULTS AND DISCUSSION

An important factor which influences enzyme activity in any biological system is pH. The results of the effect of pH of buffer on the four phosphatases studied in soils, plant materials, manures, and sewage sludges are shown in Figures 10-13. The results are given as a percentage of optimum activity because of the wide range in activity of the various materials assayed. For acid phosphatase (Figures 10 and 11), the buffer pH at which optimum activity was observed was 5.0 for all materials assayed, except soils, which showed optimum activity at pH 6.5. For alkaline phosphatase (Figures 10 and 11), the pH optima for soils and sludges were the same, being 11.0, and slightly lower in manures, being 9.5-10.5. Plant materials, however, did not contain alkaline phosphatase activity and this observation is similar to that reported by Juma (1976); he found that corn and soybean roots were lacking this enzyme. The alkaline phosphatase activity found in Canisteo and Okoboji soils, therefore, seems not to be derived from plant sources. Results obtained from assaying phosphatases in various soils showed that soils fall into two distinct groups. Soils that are acidic in nature, such as Clarion and Webster, contain primarily acid phosphatase activity, whereas neutral to alkaline soils, such as Canisteo and Okoboji, show both acid and alkaline phosphatase activity,

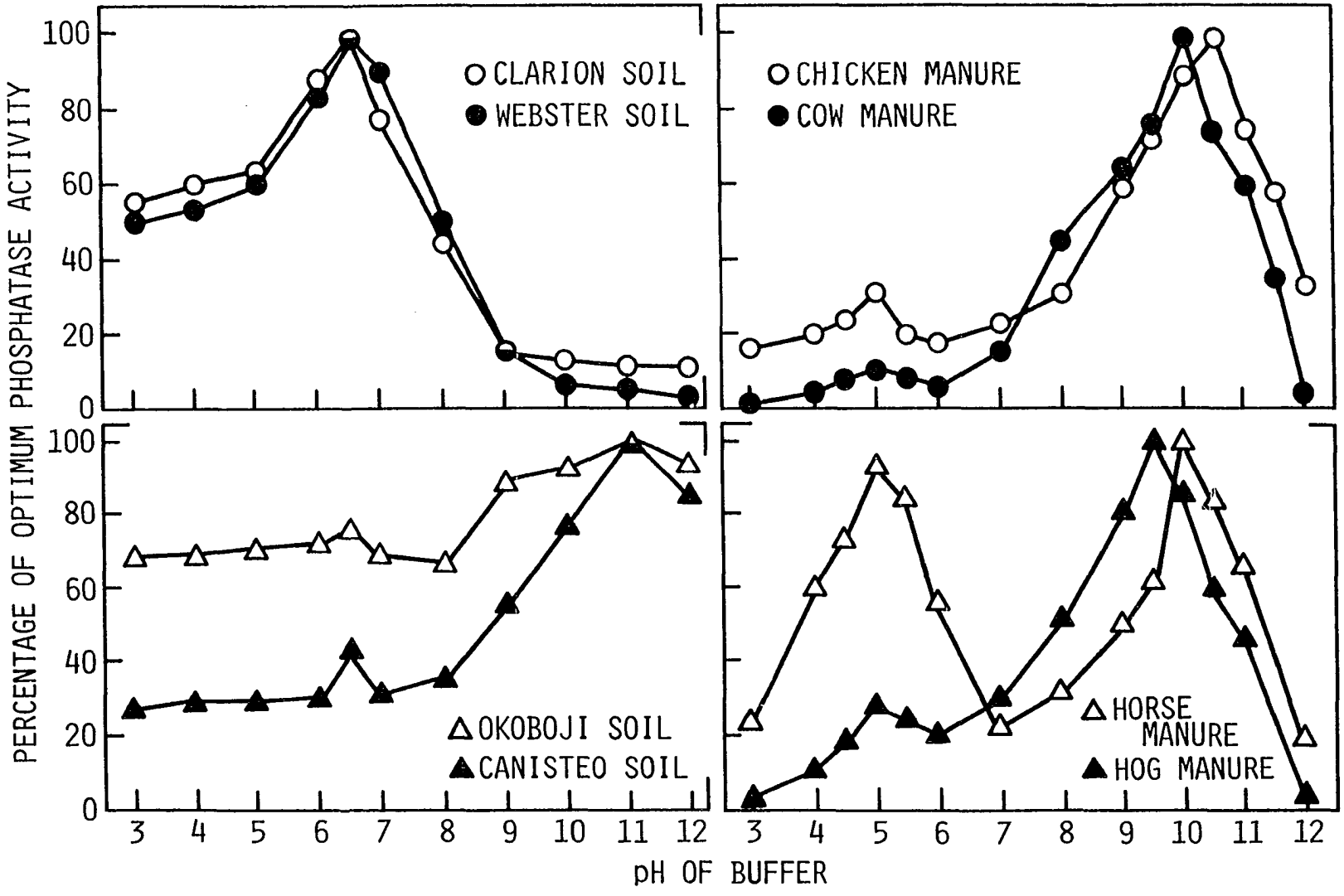


Figure 10. Effect of pH of buffer on activity of acid and alkaline phosphatases of soils and manures

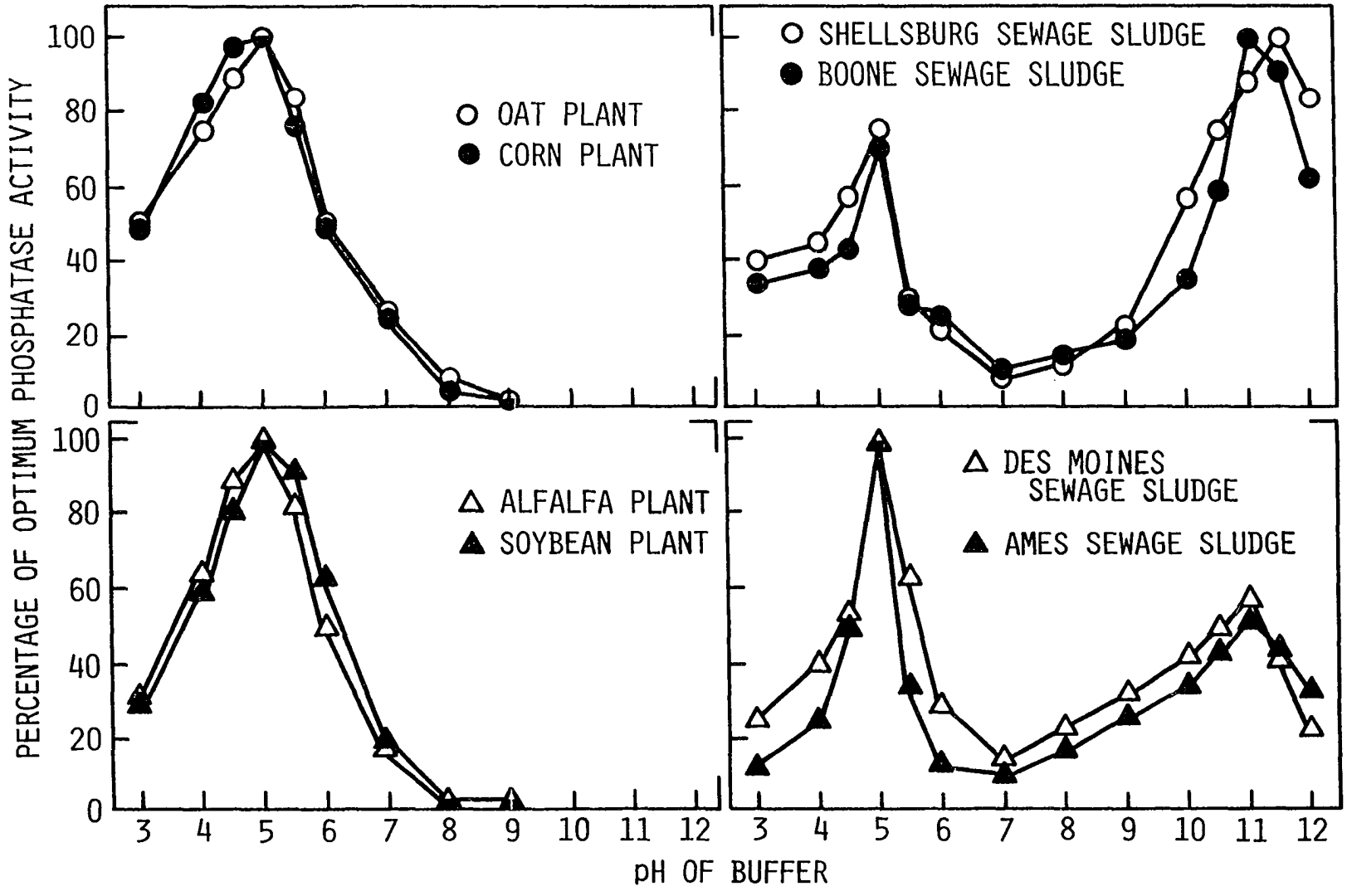


Figure 11. Effect of pH of buffer on activity of acid and alkaline phosphatases of plant materials and sewage sludges

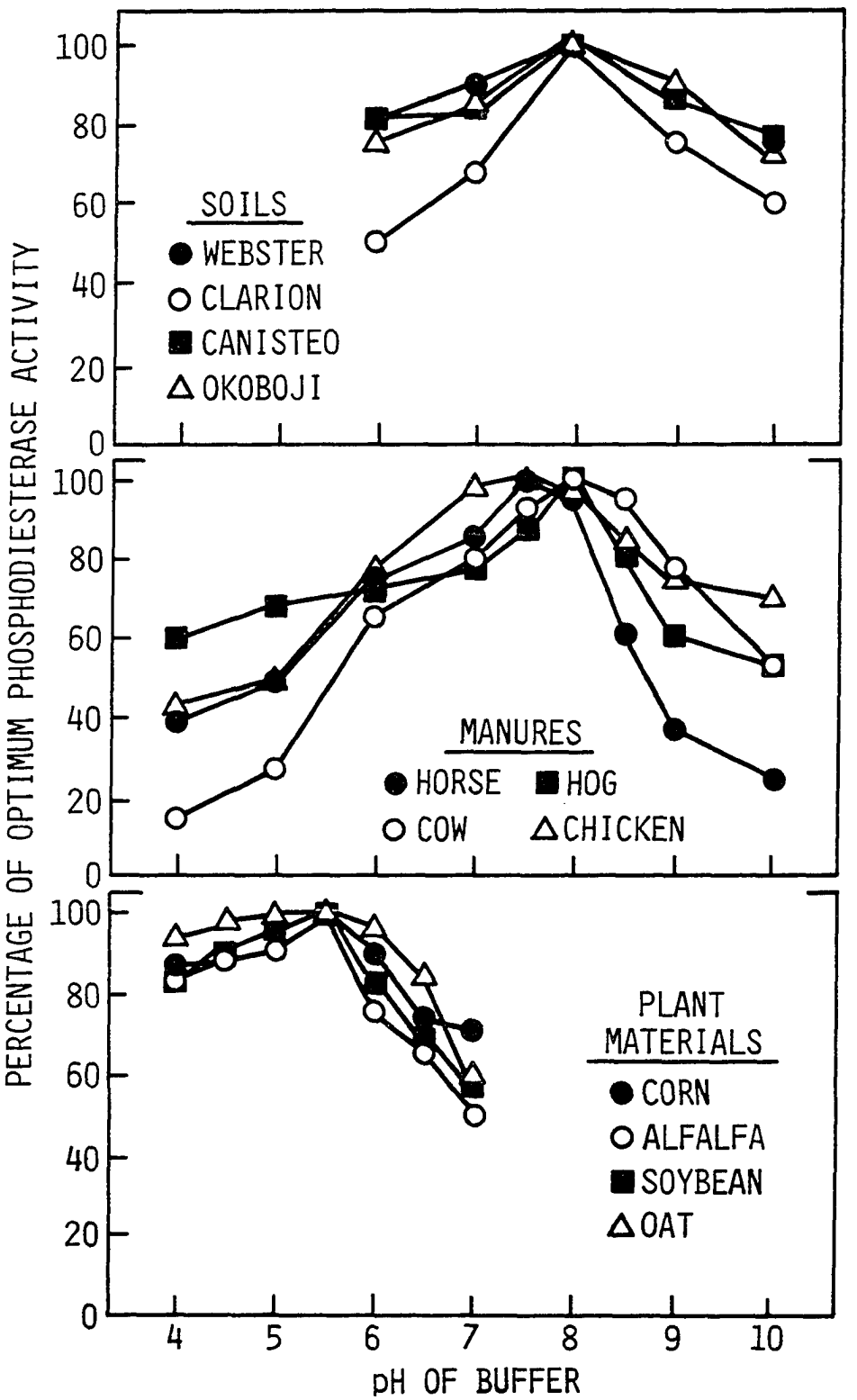


Figure 12. Effect of pH of buffer on activity of phosphodiesterase of soils, manures, and plant materials

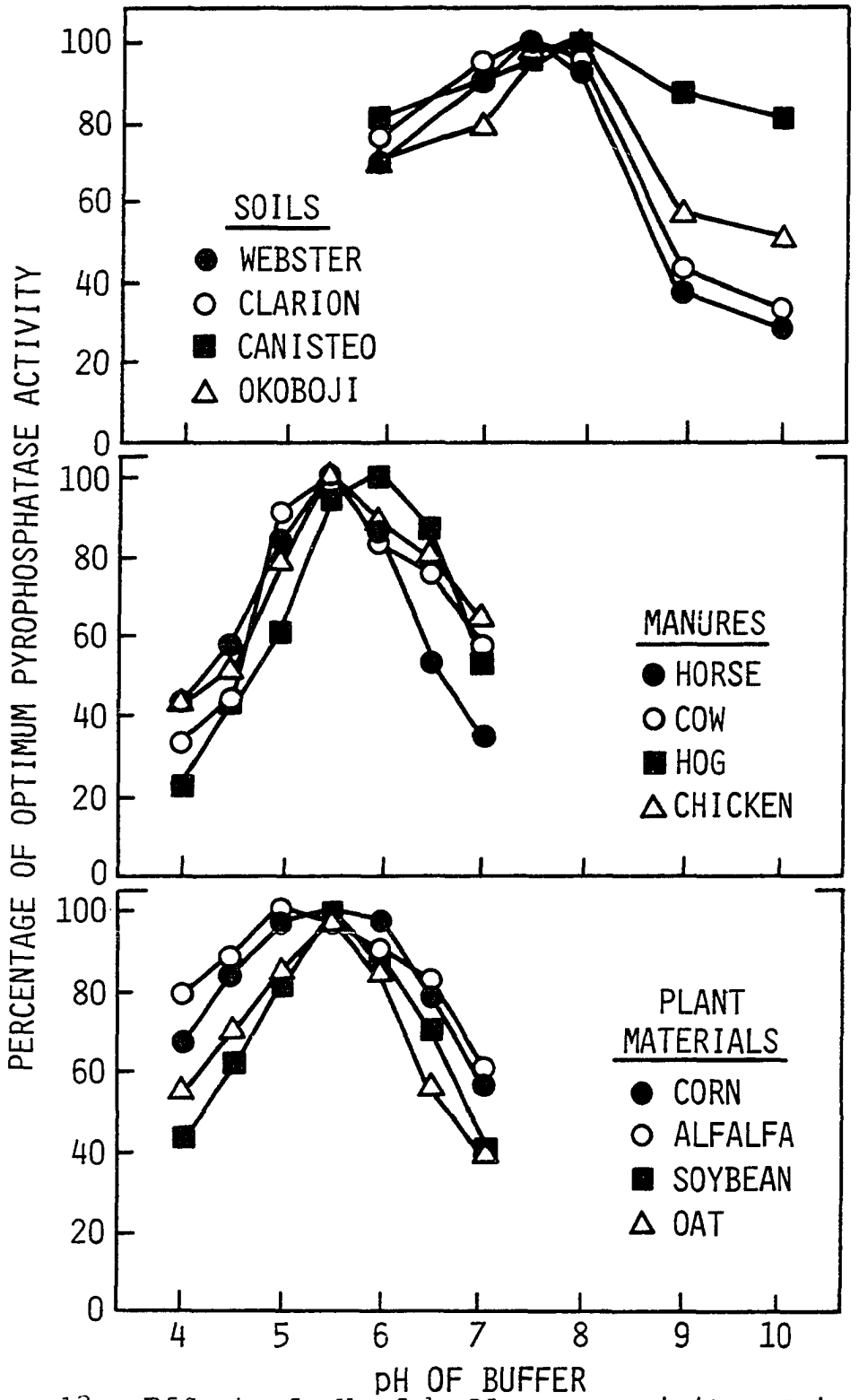


Figure 13. Effect of pH of buffer on activity of inorganic pyrophosphatase of soils, manures, and plant materials

with the latter predominating. The distribution of acid and alkaline phosphatases in soils found in this study supports the previous finding that acid phosphatase is predominate in acid soils and alkaline phosphatase is predominate in alkaline soils (Eivazi and Tabatabai, 1977; Juma and Tabatabai, 1978). Also, manures generally contained higher levels of alkaline phosphatase activity than acid phosphatase activity.

The effect of buffer pH on phosphodiesterase activity in the materials studied is shown in Figure 12. The pH optimum found for soils (8.0) was the same as that reported by Browman and Tabatabai (1978). The pH optimum for phosphodiesterase in manures ranged from 7 to 8 and in plants it was 5.5. No measureable phosphodiesterase activity was present in the sewage sludge samples used. Phosphodiesterase is the enzyme that catalyzes the hydrolytic cleavage of one of two organic moieties bound by ester linkages to phosphoric acid. An example of its role in the P cycle of soils is in the degradation of nucleic acids which are added to soils in plant litter and decaying microorganisms. Its presence in soils was previously demonstrated by Eivazi and Tabatabai (1977) and Browman and Tabatabai (1978).

For pyrophosphatase (Figure 13), the greatest activity observed for soils was at buffer pH 8.0 which is 2-3 units higher than that observed in manures (pH optimum, 5.5-6.0) and plant materials (pH optimum, 5.0-5.5). Sludges did not contain any detectable pyrophosphatase activity. Addition of

sewage sludges, therefore, seem not to directly contribute pyrophosphatase and phosphodiesterase activity to soils.

For the characterization of enzymes in soils it has been considered desirable to obtain the kinetic data (Skujins, 1976). Tables 9 and 10 compare the Michaelis constants (K_m) and the maximum rates of reaction (V_{max}) of the four phosphatases studied in soils, plant materials, manures, and sewage sludges. If an enzyme follows simple kinetics, as predicted by the Michaelis-Menten equation, $v = V_{max}S/(S + K_m)$, a rectangular hyperbola is obtained when the initial rate of velocity (v) is plotted against the substrate concentration (S). Transformation of the data to fit the double reciprocal Lineweaver-Burk equation, $1/v = (K_m/V_{max}) \cdot 1/S + (1/V_{max})$, allows calculation of the K_m and V_{max} constants by plotting 1/v vs. 1/S. The y-intercept is equal to the value of $1/V_{max}$ and the x-intercept is equal to $-1/K_m$. The K_m constant is independent of substrate concentration and can be defined in simple terms as the substrate concentration at which the reaction proceeds at one-half the maximum velocity. It is also often considered to be a measure of the affinity of the enzyme for its substrate. The V_{max} constant depends on the enzyme concentration and can be defined as the velocity obtained when the enzyme is saturated with substrate. Figure 14 shows a representative double reciprocal Lineweaver-Burk plot from which the K_m and V_{max} values for acid phosphatase in horse manure, oat plant material, Canisteo soil, and Boone

Table 9. Comparison of Michaelis constants (K_m) among the various sources of four phosphatases

Source of enzyme	Michaelis constant (mM)			
	Acid phosphatase	Alkaline phosphatase	Pyrophosphatase	Phosphodiesterase
Soils				
Clarion	1.94	2.85	47.8	1.63
Webster	1.17	4.88	38.2	1.81
Okoboji	2.46	2.63	37.8	2.16
Canisteo	3.17	1.97	27.2	1.26
Average	2.19	3.08	37.8	1.72
Plant materials				
Oat	2.73	-	6.1	1.28
Alfalfa	2.93	-	8.2	0.83
Corn	2.41	-	12.7	0.62
Soybean	3.86	-	13.5	0.68
Average	2.98	-	10.1	0.85
Manures				
Cow	3.51	2.24	8.6	2.01
Hog	3.17	6.33	4.3	0.89
Chicken	1.71	1.89	7.9	1.12
Horse	1.88	1.35	4.5	2.11
Average	2.57	2.95	6.3	1.53
Sewage sludges				
Shellsburg	3.12	0.79	-	-
Boone	2.03	1.22	-	-
Des Moines	2.97	1.77	-	-
Ames	3.92	4.66	-	-
Average	3.01	2.11	-	-

Table 10. Comparison of maximum rates of reaction (V_{max}) among the various sources of four phosphatases

Source of enzyme	Maximum rate of reaction[a]			
	Acid phosphatase	Alkaline phosphatase	Pyrophosphatase	Phosphodiesterase
Soils				
Clarion	4.56	0.94	4.35	0.38
Webster	3.46	0.87	5.40	2.94
Okoboji	3.56	4.29	2.87	4.03
Canisteo	1.35	3.28	0.69	2.07
Average	3.23	2.35	3.33	2.36
Plant materials				
Oat	767	-	548	132
Alfalfa	2,270	-	1,280	262
Corn	793	-	1,150	178
Soybean	3,700	-	3,250	315
Average	1,883	-	1,560	222
Manures				
Cow	20.9	134	309	20.5
Hog	18.9	145	1,640	4.74
Chicken	35.3	104	116	6.36
Horse	53.0	34.1	113	14.1
Average	32.0	104	545	11.4
Sewage sludges				
Shellsburg	4.27	20.4	-	-
Boone	2.95	4.33	-	-
Des Moines	3.16	15.6	-	-
Ames	4.53	4.19	-	-
Average	3.73	11.1	-	-

[a]For acid phosphatase, alkaline phosphatase, and phosphodiesterase, the V_{max} values are expressed as μmoles p-nitrophenol released$\cdot g^{-1} \cdot h^{-1}$ and for pyrophosphatase they are expressed as μmoles Pi released$\cdot g^{-1} \cdot h^{-1}$.

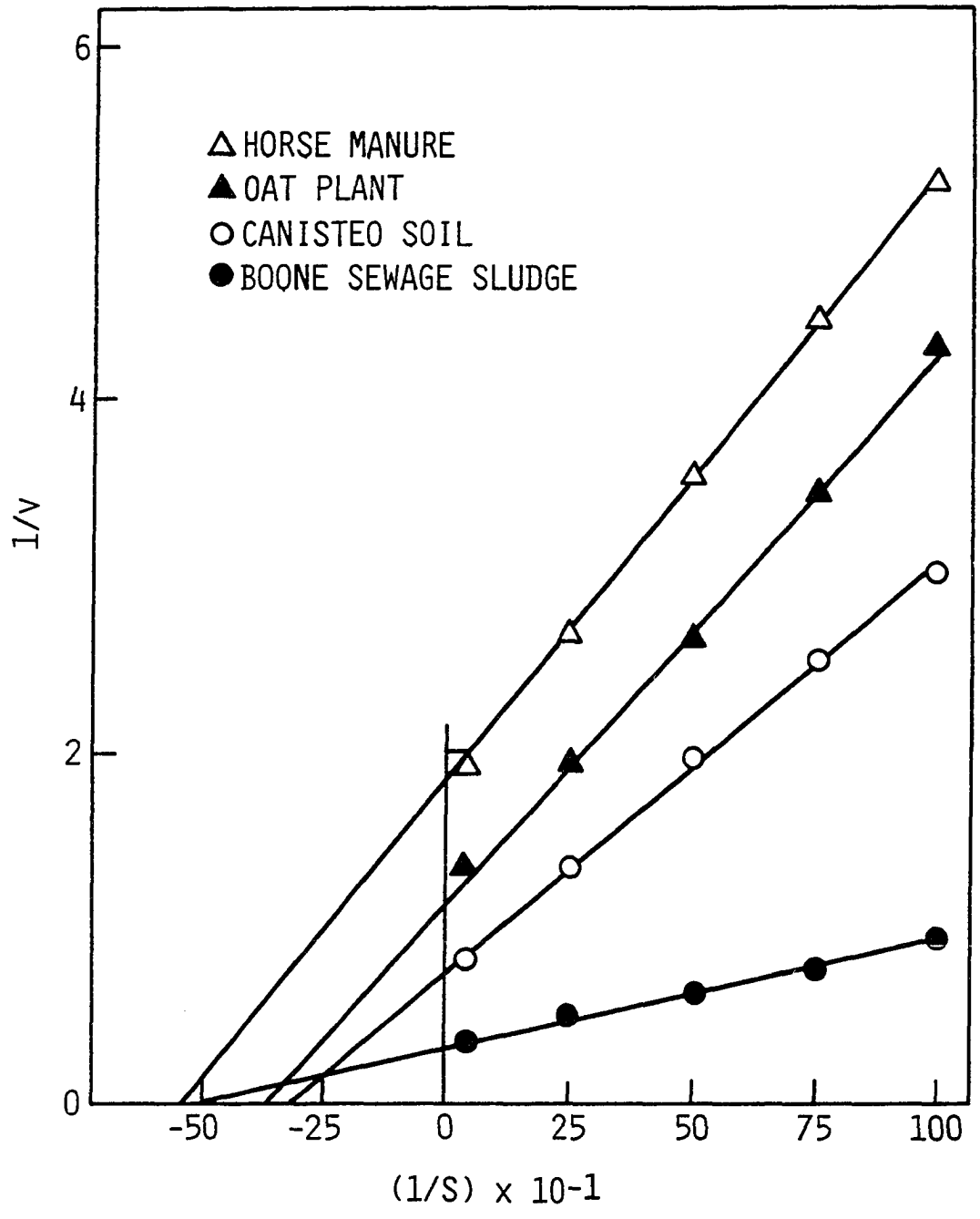


Figure 14. Double reciprocal Lineweaver-Burk plot of kinetic data for acid phosphatase activity; the initial velocity (v) is expressed as $\mu moles \cdot g^{-1} \cdot h^{-1}$ and the y axis values for horse manure are (1/v) x 10^2, for oat plant (1/v x 10^3, and for Canisteo and Boone sewage sludge, are as indicated on the graph; S is in M

sewage sludge were determined.

It should be noted that the type of buffer, the temperature of incubation, and the type of substrate used in an assay procedure can have a significant effect on the K_m value determined (Tabatabai and Bremner, 1971). However, since the assay conditions for each phosphatase studied in the soils, plant materials, manures, and sewage sludges were identical, comparison of the K_m values obtained can be made. The results indicate that the average K_m values of acid and alkaline phosphatases and phosphodiesterase in each of the four groups of samples studied (soils, plant materials, manures, and sewage sludges) were very similar (Table 9). For acid phosphatase, the average K_m values ranged from 2.19 m$\underline{M}$ for this enzyme in soils to 3.01 m$\underline{M}$ in sewage sludges. The average K_m values for alkaline phosphatase ranged from 2.11 m$\underline{M}$ for this enzyme in sewage sludges to 3.08 m$\underline{M}$ in soils, and for phosphodiesterase, the average K_m values ranged from 0.85 m$\underline{M}$ in plants to 1.72 m$\underline{M}$ in soils. However, for pyrophosphatase, the average K_m value for soils (37.8 m$\underline{M}$) was much higher than the average K_m value for this enzyme in plant materials (10.1 m$\underline{M}$) and manures (6.3 m$\underline{M}$). This high apparent K_m value of PPi for pyrophosphatase in soils compared with that for the same enzyme in plant materials and manures is partly due to sorption of PPi by soil constituents. The conformity of the reaction rates to the Lineweaver-Burk equation however, suggests that sorption of PPi by soils is propor-

tional to the PPi concentrations used.

The relative amounts of phosphatase enzyme in the materials studied can be determined by comparing the V_{max} values given in Table 10. Soils are considered enriched in enzyme protein because they exhibit more activity than can be explained by microorganism numbers (Ramirez-Martinez and McLaren, 1966). However, we found that the amount of enzyme activity in soils (when compared on a dry weight basis) is much less than that in plant materials and manures. The amount of acid phosphatase activity in the plant materials used, as indicated by the average V_{max} values, was about 580 times greater than that found in soils. On the other hand, the average activity of this enzyme in manures was about 10 times greater than that in soils. But the sewage sludges and soils seem to contain similar concentrations of this enzyme. On the average, pyrophosphatase activity in the plant materials and manures used was 470 and 160 times greater than that in soils, respectively. A similar comparison showed that the plant materials and manures contained 100 and 5 times, respectively, more phosphodiesterase than the soils studied. The plant materials used did not contain detectable amounts of alkaline phosphatase activity, but, on the average, the manure samples contained 45 times and the sewage sludges 4.7 times more alkaline phosphatase than the four soils studied (Table 10).

The amounts of plant residues annually added to soils of

the temperate region range from 1.5 to 2.0 tons per acre (1.5-2.0 mg residue/g soil) (Flaig, 1958). The contribution of plant materials to soil enzyme activity should, therefore, be significant. Foster (see Ladd, 1978), using a combination of histochemical and electron microscopic techniques, found that phosphatase activity in a light fraction of soil, fallowed for 11 months, was associated with intact cell walls of plant tissue, cell wall fragments, and amorphous organic materials. This indicates that phosphatases of plant materials may persist in soils for long periods of time. However, Zantua and Bremner (1976) found that urease activity in soils treated with waste materials (5 mg/5 g soil) initially increased, but then returned to its original level of activity after incubation for 30 days at 30 C.

Much of the enzyme activity that enters a soil system may be inhibited by organic and inorganic soil constituents, adsorbed by soil components, or degraded by soil proteases. Tables 11 and 12 show that acid phosphatase and inorganic pyrophosphatase of corn root homogenate were strongly inhibited by addition to 12 soils. The degree of inhibition of these phosphatases varied among the soils used. The nature of this inhibition remains to be investigated.

Decomposition of organic materials by microorganisms is also very rapid, with numerous bacteria, actinomycetes, and fungi capable of utilizing soluble compounds and proteins. As stated by Gray (1970), "Microorganisms live in the soil

Table 11. Inhibition of corn root acid phosphatase added to soils

Soil	Soil activity[a]	Corn root activity[b]	Combined activity[c]	Inhibition of corn root activity (%)
Ackmore	0.58	1.10	1.16	47.2
Canisteo	0.37	1.10	0.80	60.9
Clarion	0.36	1.10	0.89	51.8
Downs	0.50	1.10	1.08	47.5
Fayette	0.57	1.10	1.14	47.8
Harps	0.41	1.10	0.88	57.0
Lester	0.66	0.98	1.22	42.7
Muscatine	0.91	0.98	1.41	49.2
Nicollet	0.79	0.98	1.31	47.0
Okoboji	1.09	0.98	1.51	57.3
Tama	0.94	0.98	1.41	52.2
Webster	1.17	0.98	1.63	53.0

[a] μmoles p-nitrophenol released·g soil^{-1}·h^{-1}.

[b] μmoles p-nitrophenol released·20 mg corn root^{-1}·h^{-1}.

[c] Activity of soil and corn root homogenate when mixed together and incubated. Activity is expressed as μmoles p-nitrophenol released·g soil^{-1}·h^{-1}

Table 12. Inhibition of corn root inorganic pyrophosphatase added to soils

Soil	Soil activity[a]	Corn root activity[b]	Combined activity[c]	Inhibition of corn root activity (%)
Ackmore	1.68	1.42	2.71	37.3
Canisteo	0.36	1.42	1.29	34.1
Clarion	1.91	1.42	2.52	56.8
Downs	1.45	1.42	2.71	11.4
Fayette	1.23	1.42	1.91	52.3
Harps	0.32	1.42	0.87	61.4
Lester	1.36	1.78	2.16	54.5
Muscatine	1.97	1.78	2.81	52.7
Nicollet	1.23	1.78	1.91	61.8
Okoboji	1.07	1.78	2.16	38.2
Tama	2.00	1.78	3.04	41.8
Webster	2.36	1.78	3.58	30.9

[a] μmoles Pi released·g soil^{-1}·h^{-1}.

[b] μmoles Pi released·20 corn root^{-1}·h^{-1}.

[c] Activity of soil and corn root homogenate when mixed together and incubated. Activity is expressed as μmoles Pi released·g soil^{-1}·h^{-1}.

under starvation conditions," so that addition of an energy source greatly stimulates microbial growth in soils. Thus, the addition of organic material containing phosphatase activity is balanced by decomposition and inhibition of the enzyme protein added to soil or already present in the soil. It is of interest to note that the average V_{max} values for each phosphatase, in the four soils studied, were very similar even though the amount of the individual phosphatases added to the soil environment may vary greatly (Table 10).

Another important parameter affecting enzyme catalyzed reactions is temperature. The rate of enzyme catalysis increases with increasing temperature until at some temperature the rate begins to decrease because of inactivation of the enzyme. The temperature dependence of the rate of an enzyme reaction, below the inactivation temperature, can be described by the Arrhenius equation, $k = A \cdot \exp(-E_a/RT)$, where k is the rate constant, A is the pre-exponential factor, E_a is the activation energy, R is the gas constant, and T is the temperature in degrees K. The log form of the Arrhenius equation is given as $\log k = (-E_a/2.303RT) + \log A$, and is useful for calculating E_a by plotting log k or log of initial rate against 1/T. A representative Arrhenius equation plot for acid phosphatase activity in corn plant, horse manure, Webster soil, and Ames sewage sludge is shown in Figure 15. From the slope of the line, which is equal to $-E_a/2.303\ RT$, we can calculate the energy of activation. Comparison of the

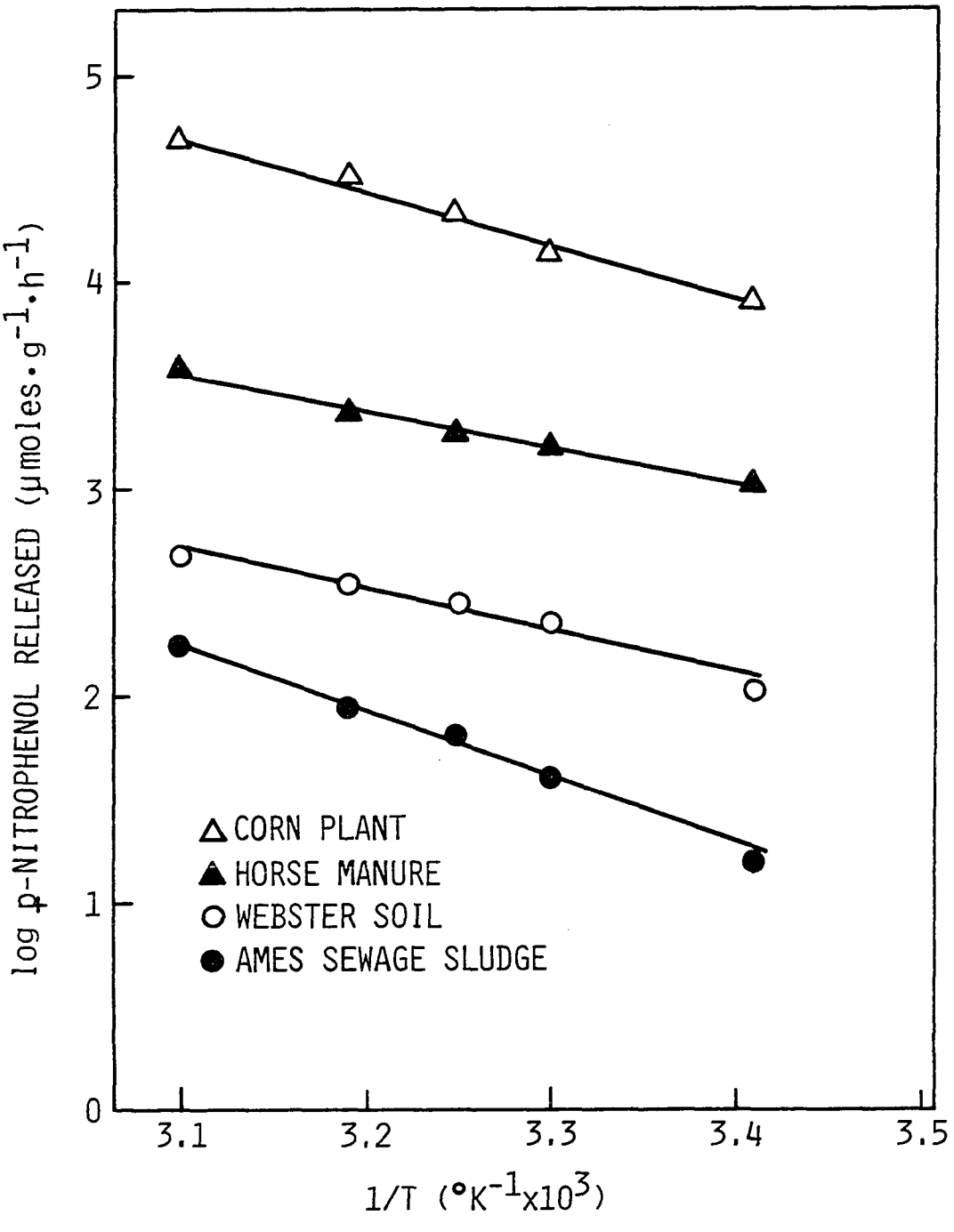


Figure 15. Arrhenius equation plot of acid phosphatase activity values

results for the four phosphatases show that the average E_a values, expressed in kJ/mole, for each enzyme within the different materials assayed were very similar, with the exception of the acid phosphatase in the sewage sludges (average E_a value, 50.6 kJ/mole) (Table 13). The lowest average E_a value among the other soils and organic waste materials studied was observed for alkaline phosphatase in soils (32.3 kJ/mole); the highest was for acid phosphatase in soils (42.3 kJ/mole).

A more direct attempt to determine the effects that materials such as plants, manures, and sewage sludges may have on increasing phosphatase activity in soils is reported in the second part of this study. A long-term incubation experiment which included 10 different treatments applied to Webster or Canisteo soil was designed. The chemical properties of the soils and the materials added to the soils are shown in Table 8. Webster soil is slightly acid in nature (soil pH 6.5) and contained much more phosphatase activity than did the calcareous Canisteo soil (soil pH 7.7). The amount of material added (3 mg/g soil which is equivalent to 3 tons/acre) was designed to approximate levels that are normally added to soils on an annual basis (Flaig, 1958). Soils were wetted with 0.5 ml deionized water so that moisture would not be lacking during microbial growth. In addition, steam sterilized materials were included in the treatments to aid in determining the immediate effect of these materials on increasing phosphatase activity in soils. Any increase in activity

Table 13. Comparison of energies of activation (E_a) among the various sources of four phosphatases

Source of enzyme	Energy of activation (kJ/mole)			
	Acid phosphatase	Alkaline phosphatase	Pyrophosphatase	Phosphodiesterase
Soils				
Clarion	41.9	30.2	35.0	42.3
Webster	42.2	34.2	32.9	36.9
Okoboji	43.5	33.8	43.2	32.8
Canisteo	41.5	30.8	39.3	36.0
Average	42.3	32.3	37.6	37.0
Plant materials				
Oat	41.0	-	31.8	36.3
Alfalfa	41.7	-	39.0	44.8
Corn	46.1	-	36.6	42.6
Soybean	35.4	-	35.7	42.7
Average	41.1	-	35.8	41.6
Manures				
Cow	38.3	31.5	43.7	35.9
Hog	35.3	39.9	41.2	46.9
Chicken	30.4	31.0	36.3	44.9
Horse	32.4	40.8	38.9	39.5
Average	34.1	35.8	40.0	41.8
Sewage sludges				
Shellsburg	38.4	41.0	-	-
Boone	53.7	47.3	-	-
Des Moines	51.5	36.6	-	-
Ames	58.6	43.5	-	-
Average	50.6	42.1	-	-

observed in soil treated with material not steam sterilized over that treated with steam sterilized material would be due to contribution of the enzyme in the material added. Treatments containing sodium azide were also included because it has been suggested as being useful in the study of extracellular enzymes in soils. Azides can inhibit the proliferation of microorganisms and thus the synthesis of new enzymes (Skujins, 1976). Azides have also been found to be potentially useful agents in controlling plant diseases and as a herbicide against a variety of weeds (Parochetti and Warren, 1970). Information is needed, however, on the effect of azides on soil phosphatases.

The results obtained on the effect of the organic waste materials on acid phosphatase and pyrophosphatase in two soils (Webster and Canisteo) are shown in Figures 16-18 and 19-21, respectively. The analyses of variance for the activity of these two enzymes as affected by soil, soil treatment, and time during the incubation experiment are shown in Tables 14 and 15. The main effects of soil, treatment, and time on acid phosphatase and pyrophosphatase activity were significant at the 0.1% level. The two factor interaction effects on acid phosphatase activity were also significant at the 0.1% level (Table 14). However, for pyrophosphatase activity, the treatment x soil and the soil x time interaction effects were significant at the 0.1% significance level, but the treatment x time interaction effect was significant at the 5% level

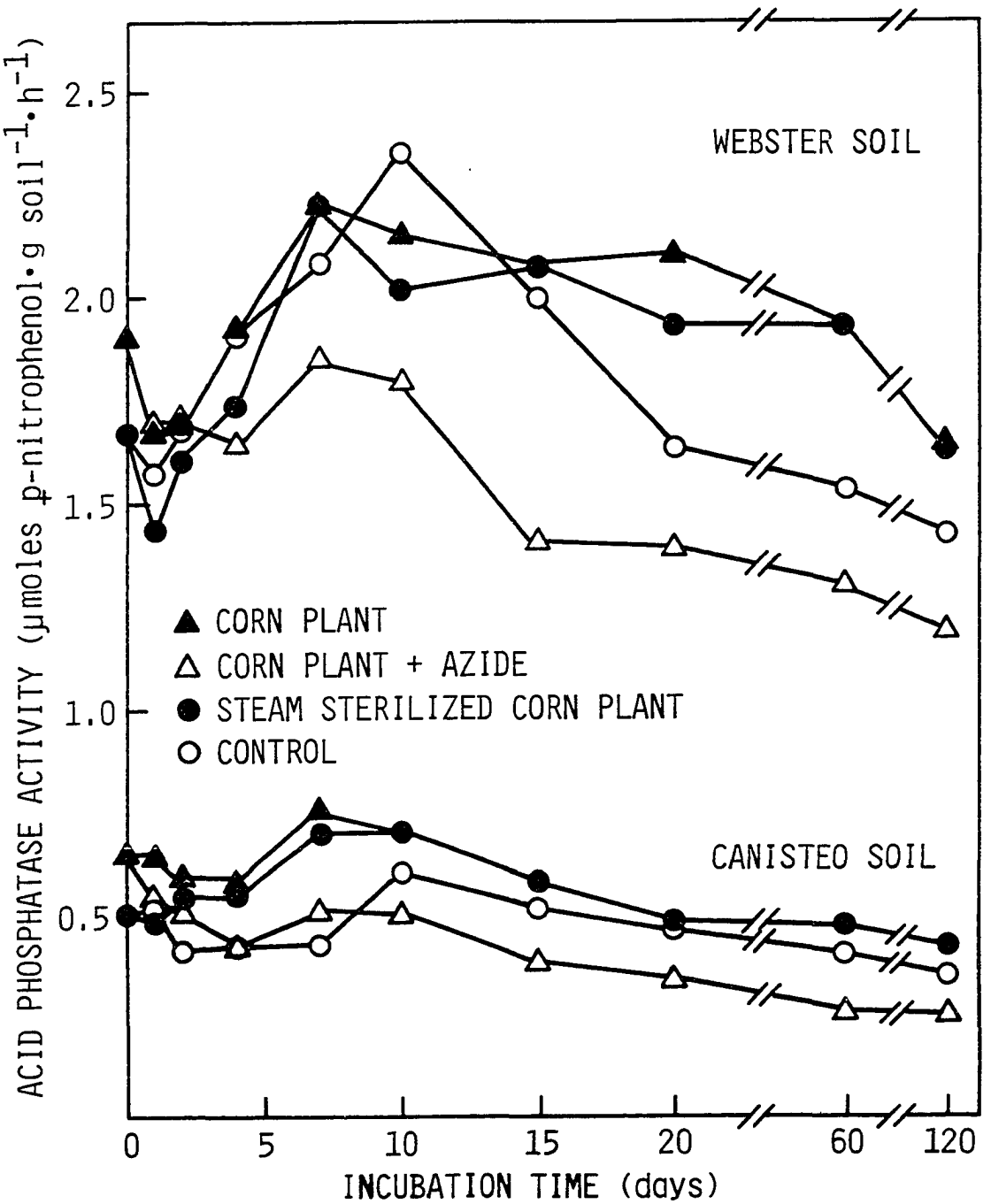


Figure 16. Changes in acid phosphatase activity in Webster and Canisteo soils as influenced by addition of corn plant material, corn plant material + azide, or steam sterilized corn plant material during 120 days of incubation

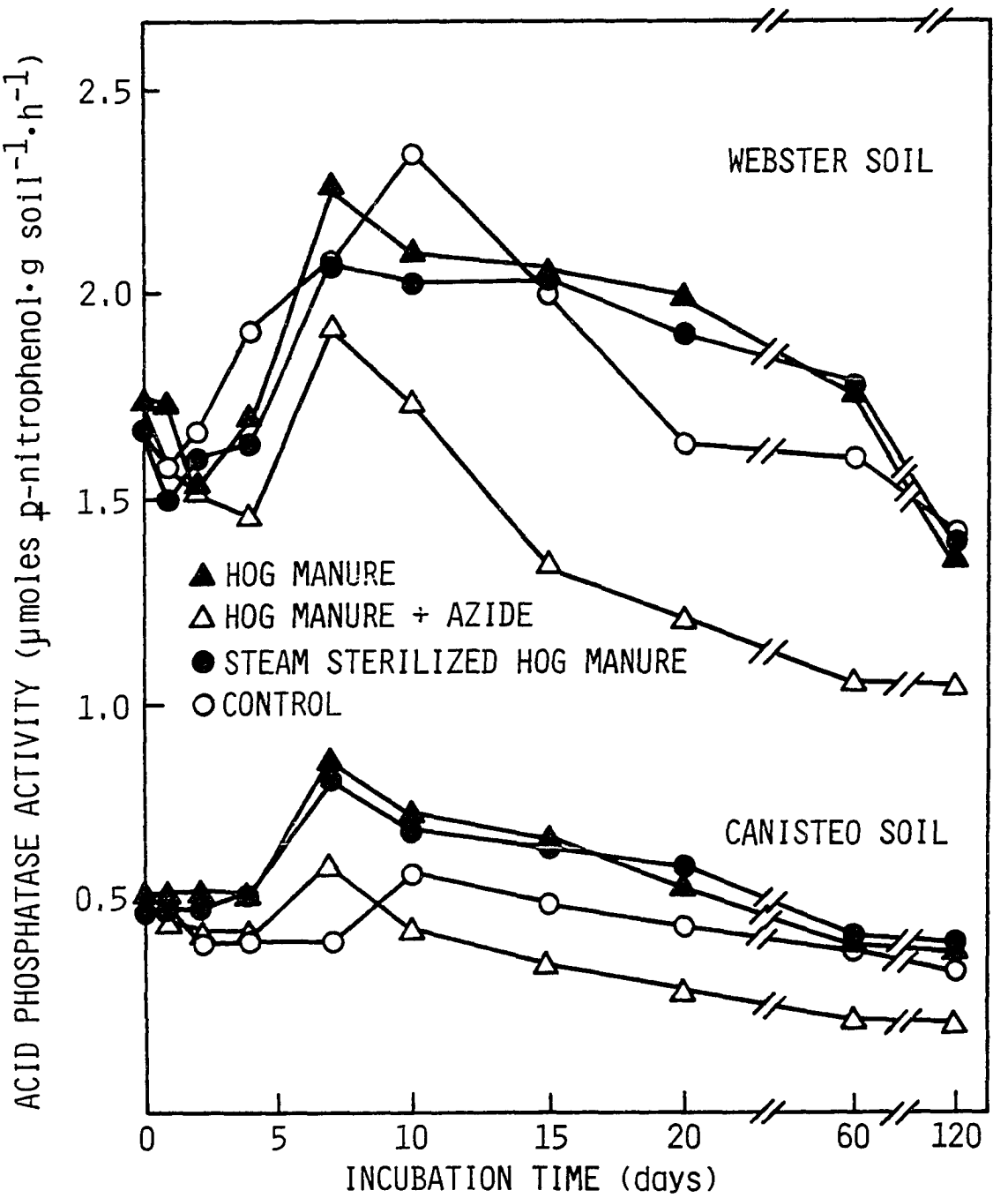


Figure 17. Changes in acid phosphatase activity in Webster and Canisteo soils as influenced by addition of hog manure, hog manure + azide, or steam sterilized hog manure during 120 days of incubation

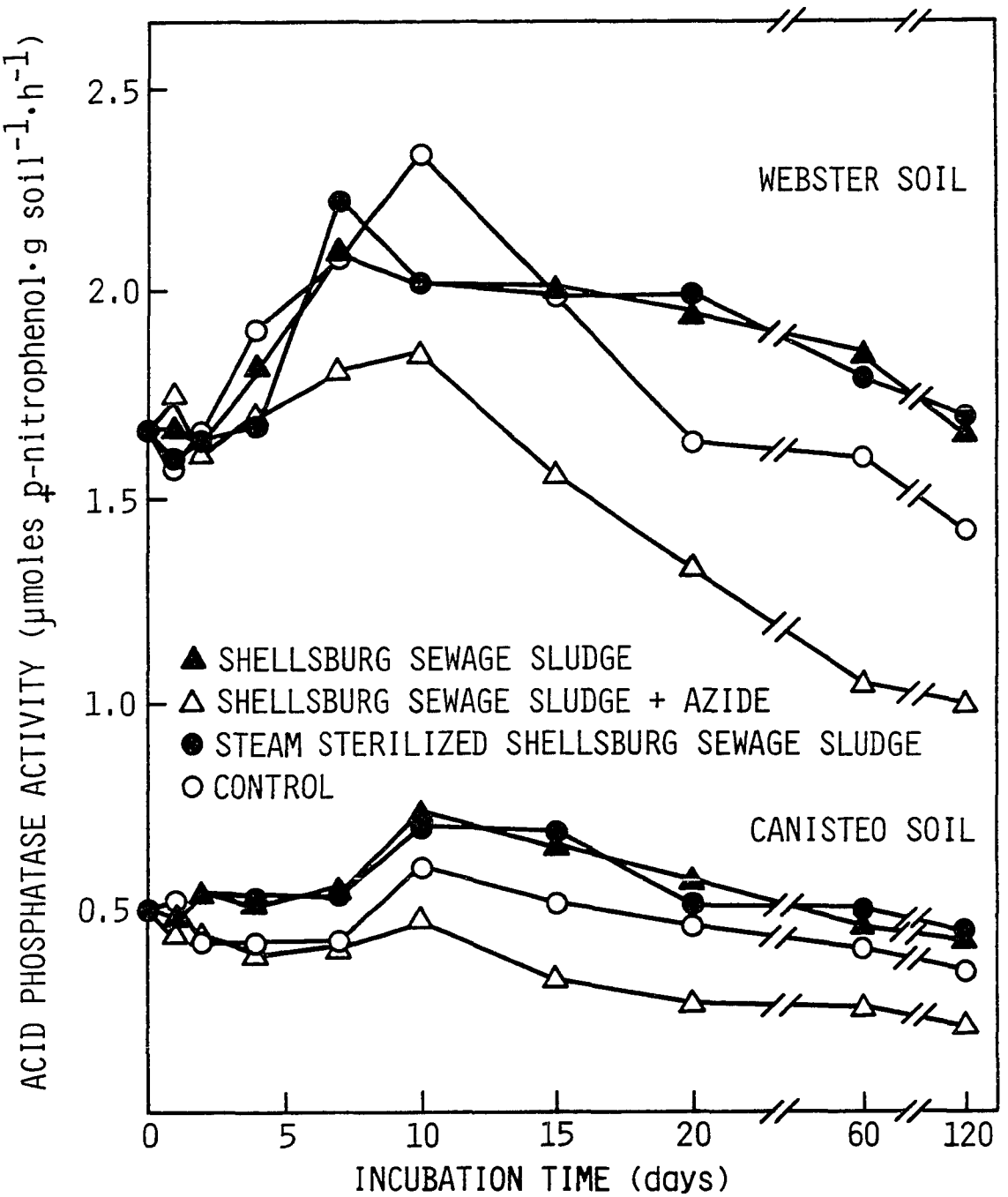


Figure 18. Changes in acid phosphatase activity in Webster and Canisteo soils as influenced by addition of Shellsburg sewage sludge, Shellsburg sewage sludge + azide, or steam sterilized Shellsburg sewage sludge during 120 days of incubation

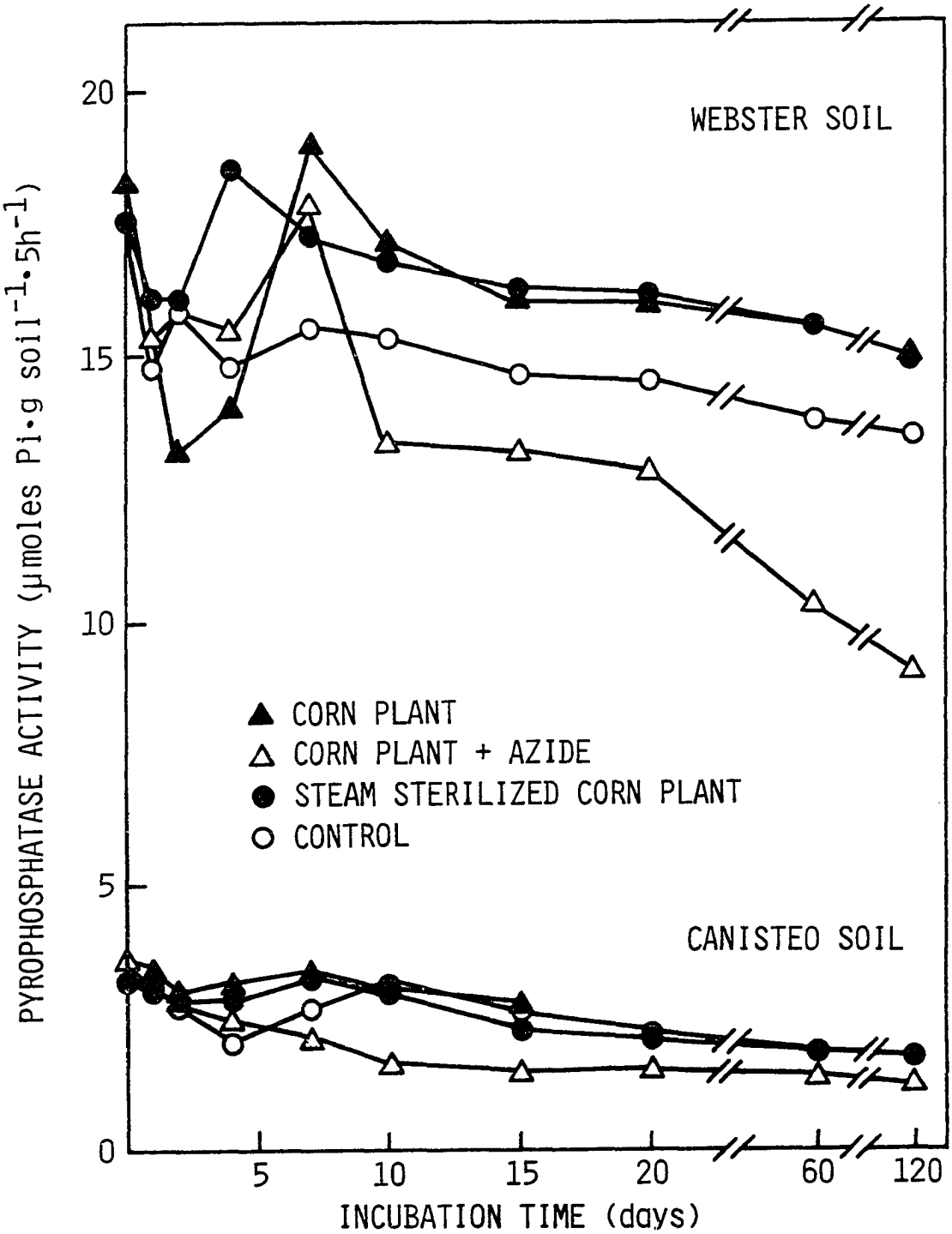


Figure 19. Changes in pyrophosphatase activity in Webster and Canisteo soils as influenced by addition of corn plant material, corn plant material + azide, or steam sterilized corn plant material during 120 days of incubation

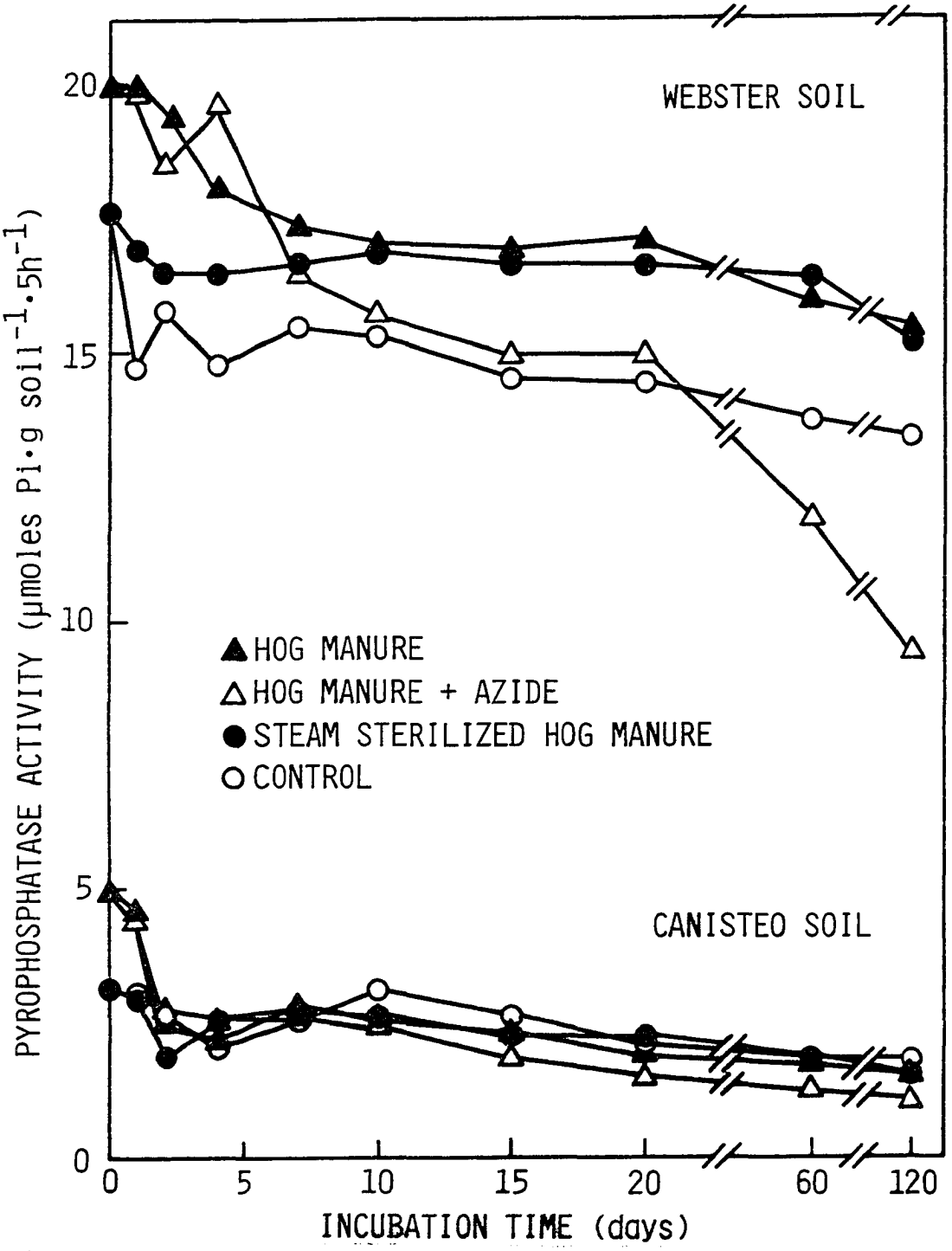


Figure 20. Changes in pyrophosphatase activity in Webster and Canisteo soils as influenced by addition of hog manure, hog manure + azide, or steam sterilized hog manure during 120 days of incubation

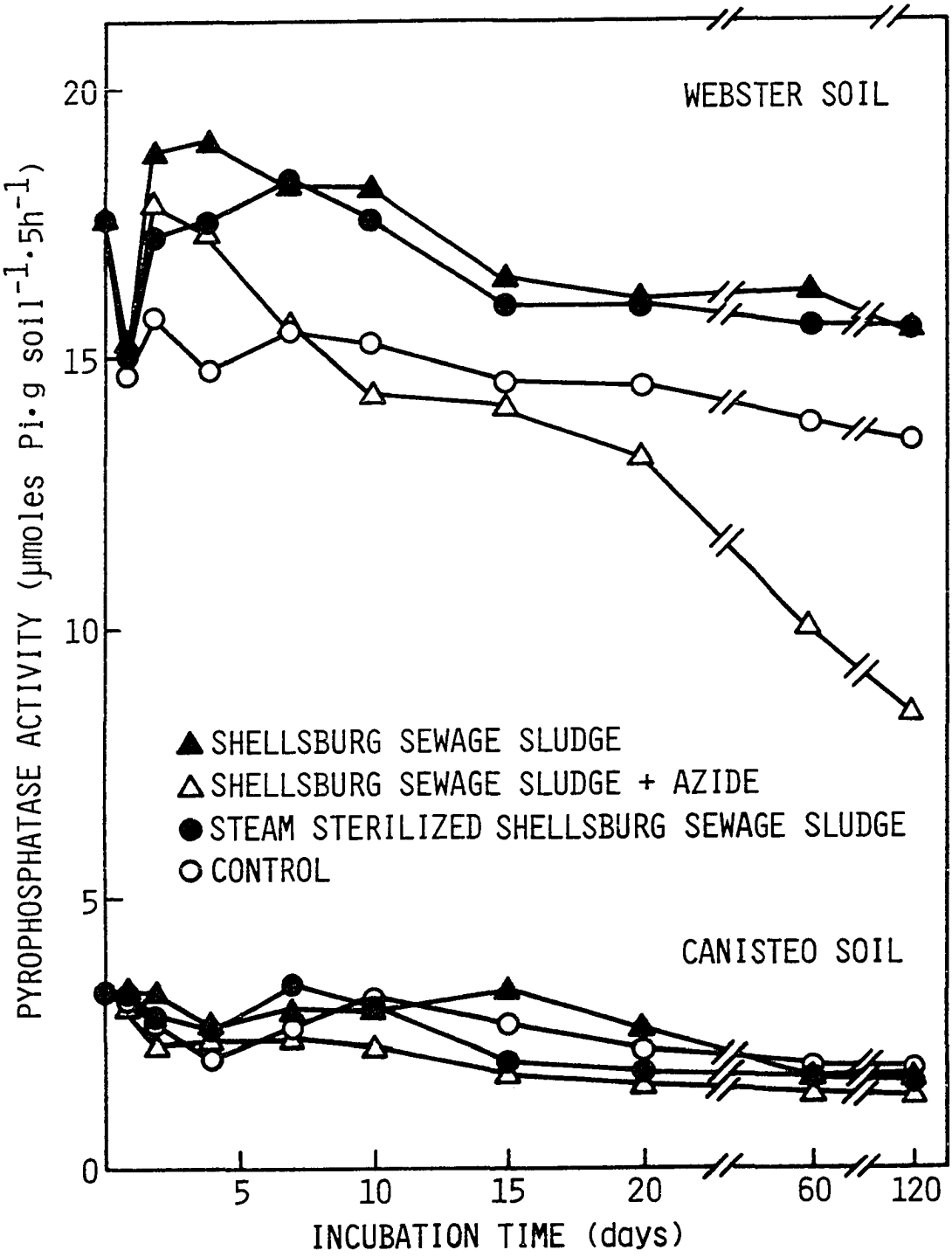


Figure 21. Changes in pyrophosphatase activity in Webster and Canisteo soils as influenced by addition of Shellsburg sewage sludge, Shellsburg sewage sludge + azide, or steam sterilized Shellsburg sewage sludge during 120 days of incubation

Table 14. Analyses of variance for acid phosphatase activity values as affected by soil, soil treatment, and time

Source of variance	d.f.	Mean square
Soil	1	91.1***
Treatment	9	0.402***
Treatment x soil	9	0.076***
Time	11	0.356***
Soil x time	11	0.069***
Treatment x time	99	0.021***
Remainder	99	0.0097

***Significant at the 0.1% level.

Table 15. Analyses of variance for inorganic pyrophosphatase activity values as affected by soil, soil treatment, and time

Source of variance	d.f.	Mean square
Soil	1	10,700***
Treatment	9	14.2***
Treatment x soil	9	8.22***
Time	11	24.5***
Soil x time	11	4.75***
Treatment x time	99	1.43*
Remainder	99	1.00

*Significant at the 5% level.

***Significant at the 0.1% level.

(Table 15). Differences at the 5% significance level, between the overall treatment means (Table 16) and the overall incubation time means (Table 17), for acid phosphatase and pyrophosphatase, were determined by Duncan's multiple range test.

Analyses of the acid phosphatase and pyrophosphatase activities measured during the incubation experiment showed that the addition of corn plant material to soils increased soil phosphatase activity at zero time of incubation. This is seen by examining Figure 16 where activity, at zero time of incubation, of soil treated with corn plant material not steam sterilized was significantly higher ($P<0.05$) than soil treated with steam sterilized corn plant material. Hog manure gave a slight increase in acid phosphatase activity at zero time of incubation (Figure 17), but it was not significant at the 5% level. No significant effect on activity of this enzyme was observed at zero time of incubation when sewage sludge was added to soils (Figure 18). For pyrophosphatase, however, corn plant material and sewage sludge did not contribute significantly to the activity of this enzyme in soils at zero time of incubation (Figures 19 and 21). The pyrophosphatase activity observed in the soil samples treated with hog manure not steam sterilized was found to be significantly higher ($P<0.05$) than the activity of soil samples treated with steam sterilized hog manure (Figure 20). This difference between the soil samples remained significant after one day of incubation.

Table 16. The effect of various treatments on acid phosphatase and inorganic pyrophosphatase activity of soil

Enzyme	Treatment[a]	Treatment mean[b]
Acid phosphatase	Control	1.10 c
	Corn plant	1.25 a
	Corn plant (SS)	1.18 b
	Corn plant (N_3)	0.97 d
	Shellsburg sewage sludge	1.18 b
	Shellsburg sewage sludged (SS)	1.17 b
	Shellsburg sewage sludge (N_3)	0.91 e
	Hog manure	1.19 b
	Hog manure (SS)	1.16 b
	Hog manure (N_3)	0.89 e
Inorganic pyrophosphatase	Control	8.64 d
	Corn plant	9.28 bc
	Corn plant (SS)	9.41 bc
	Corn plant (N_3)	7.79 e
	Shellsburg sewage sludge	9.78 ab
	Shellsburg sewage sludge (SS)	9.43 bc
	Shellsburg sewage sludge (N_3)	7.84 e
	Hog manure	10.09 a
	Hog manure (SS)	9.43 bc
	Hog manure (N_3)	8.96 cd

[a]SS, soil treated with steam sterilized material; N_3, soil treated with 0.5 ml 0.05% sodium azide.

[b]Treatment means for acid phosphatase are expressed as μmoles p-nitrophenol released·g soil^{-1}·h^{-1} and for inorganic pyrophosphatase the means are expressed as μmoles Pi released·g soil^{-1}·5 h^{-1}. Treatment means, for each phosphatase, followed by the same letter do not differ significantly at the 5% level (Duncan's multiple range test).

Table 17. The effect of incubation time on acid phosphatase and inorganic pyrophosphatase activity of soil

Enzyme	Incubation time (days)	Incubation time mean[a]
Acid phosphatase	0	1.12 c
	1	1.06 c
	2	1.06 c
	4	1.10 c
	7	1.34 a
	10	1.31 a
	15	1.20 b
	20	1.10 c
	30	1.07 c
	60	0.99 d
	90	0.97 d
	120	0.88 e
Inorganic pyrophosphatase	0	10.97 a
	1	9.97 b
	2	9.77 b
	4	9.78 b
	7	10.01 b
	10	9.41 bc
	15	8.86 cd
	20	8.64 d
	30	8.51 de
	60	7.91 ef
	90	7.58 f
	120	7.35 f

[a]Incubation time means for acid phosphatase are expressed as μmoles p-nitrophenol released·g soil^{-1}·h^{-1} and for inorganic pyrophosphatase the means are expressed as μmoles Pi released·g soil^{-1}·5 h^{-1}. Incubation time means, for each phosphatase, followed by the same letter do not differ significantly at the 5% level (Duncan's multiple range test).

Generally, both acid phosphatase and pyrophosphatase activity in the samples treated with waste materials decreased after one day of incubation (Figures 16-21). This decrease was especially evident for pyrophosphatase activity in Webster soil (Figures 19-21). A decrease in activity for both of these enzymes in soil samples not treated with the waste materials was also evident. The results obtained for the untreated soil samples are consistent with those obtained by Zantua and Bremner (1977) for urease. They reported an initial decrease in activity when air-dried soils were rewetted and incubated. A similar decrease was not observed, however, when field-moist soils were incubated. Rewetting of air-dried soils causes a breakdown of aggregates in the soils (Tabatabai and Bremner, 1970). The breakdown of the aggregates in the soil samples studied in this work is thought to increase the susceptibility of the phosphatase enzymes to inhibition by soil components or degradation by soil microorganisms. The similar decrease in the samples treated with organic waste materials suggests that such a phenomenon is occurring in these samples, also.

After one week incubation, acid phosphatase and pyrophosphatase activities increased, due to microbial growth. The highest average acid phosphatase activity was observed after 7-10 days incubation (Table 17). A similar increase in pyrophosphatase activity was observed after 7 days of incubation but this increase could not make up for the initial

loss in activity that occurred when the air-dried soils were rewetted. The overall means at the various incubation times indicate that activity was significantly decreased during the course of incubation (Table 17). However, further investigation revealed that this decrease was due primarily to the effect of sodium azide and only those samples treated with azide showed a significant decrease ($P<0.05$) in activity. The other treatments, such as corn plant material, hog manure, and sewage sludge not treated with sodium azide, showed a slight trend towards increasing acid phosphatase activity after 120 days of incubation. This increase in activity, however, was not significant at the 5% level. Acid phosphatase and pyrophosphatase activity observed in the soil samples after 120 days incubation were not affected by change in soil pH. This is because a less than 0.2 pH unit change was observed in the soils after 120 days of incubation, from that observed at zero time of incubation.

Additions of organic materials, such as those used in this study, may lead to increases in phosphatase activity in soils. Spier and Ross (1978), however, theorized that a great many flushes of microbial growth and activity, resulting from such additions, are required to cause a permanent increase in the level of phosphatase activity in soils.

The decrease in activity observed for acid phosphatase and pyrophosphatase when treated with sodium azide was very striking and deserves special recognition. Sodium azide

treated soils (500 ppm treatment level) also showed a slight increase in activity after 7-10 days incubation (Figures 16-21), but it was not as pronounced as that observed for the other treatments. Although sodium azide was not absolutely effective in preventing microbial proliferation, it did seem to inhibit it as can be seen by the lower activity for acid phosphatase and pyrophosphatase in azide-treated samples during the peak of microbial growth (after 7-10 days) when compared to the other treatments. Comparison of the overall treatment means showed a significant difference ($P<0.05$) between the azide-treated samples and azide-untreated samples, with azide causing a decrease in activity (Table 16). Further investigation indicated that this significant decrease in activity began after 15 days of incubation and remained significant throughout the remainder of the 120 day incubation period.

Azides are inhibitors of metal-containing enzymes, and their effect on soil enzymatic activity (invertase and amylase) and on soil microbial populations was investigated by Kelly and Rodriguez-Kabana (1975). They added increasing amounts of potassium azide (0-224 kg/ha) to field plots and measured enzymatic activity and microbial population levels after 2, 5, and 16 weeks. Kelly and Rodriguez-Kabana (1975) found that, as the azide rates increased, the microbial population levels decreased when measurements were made after 2 weeks. After 16 weeks, however, there was little difference

between the treatments and control. A decline in invertase and amylase was also observed at the 2-week sampling date and remained at this level even though microbial numbers had increased to control levels. Kelly and Rodriguez-Kabana (1975) concluded that the new populations of microorganisms produced in the azide treated soils lacked species possessing significant amounts of invertase and amylase activity.

The possibility that azide affects extracellular enzyme activity was also considered. However, in the work reported, it was found that sodium azide did not affect acid phosphatase and pyrophosphatase activity when added to soils immediately before assay. Thus, it seems that azide has no effect on activity of native phosphatases in the soil environment. The loss in phosphatase activity observed during incubation, therefore, must be due to inhibition of microbial proliferation or lack of species possessing significant amounts of phosphatases. The decrease in activity observed in the soils treated with sodium azide remained significant after 120 days of incubation. It is uncertain how long the phosphatase activity in such samples would lag behind those of azide-untreated samples had the experiment continued for longer times.

It is concluded that organic materials added to soils play a significant role in maintaining levels of phosphatase activity by allowing microbial activity to occur in the soil environment. This activity results in the microorganisms

releasing extracellular enzymes into the soil and also provides phosphatase activity in soils through enzymes associated with living and dead microbial cells.

PART IV. INHIBITION OF CORN ROOT ACID PHOSPHATASE AND INORGANIC PYROPHOSPHATASE BY CLAY MINERALS

INTRODUCTION

The phosphatases present in the soil environment play a significant role in P availability to plants from polyphosphate fertilizers and native soil organic P compounds (Kiss et al., 1975). The precise physical state of the phosphatases in soils is not yet clearly understood, but it is apparent that, in general, enzymes are adsorbed on colloidal soil particles, such as clay minerals, or incorporated in the clay-humus complexes.

Adsorption of proteins by clay minerals has been investigated by several workers in the past 40 years (Ensminger and Gieseking, 1941, 1942; McLaren and Estermann, 1956; Armstrong and Chesters, 1964; Albert and Harter, 1973). These investigations were concerned primarily with the reactions between clay minerals and the proteins with respect to (1) the amounts of protein that can be adsorbed, (2) the conditions under which the protein is adsorbed, (3) the effect of the type of clay mineral used in the reaction, (4) the expansion of the clay lattice resulting from protein adsorption, and (5) the stability of the clay-adsorbed protein to microbial degradation (Armstrong and Chesters, 1964).

The adsorption of pepsin and lysozyme by Mg-Wyoming bentonite (mainly montmorillonite) was studied by Armstrong and Chesters (1964). They reported that adsorption of these proteins was rapid, with 90% of maximum adsorption occurring

within 3 min of equilibration. The maximum adsorption of pepsin and lysozyme was found to occur at pH values close to the isoelectric points of these proteins. In addition, x-ray diffraction analyses of protein-bentonite complexes showed that adsorption of lysozyme resulted in an expansion of the clay lattice.

Albert and Harter (1973) studied the adsorption of lysosyme and ovalbumin by four Na-clay minerals (smectite, biotite-vermiculite, illite, and kaolinite). They found that adsorption of these proteins was influenced by the clay suspension pH, adsorption increasing as the clay suspension pH approached the protein isoelectric point. Adsorption of lysozyme and ovalbumin also caused an increase in sodium ion concentration in the clay suspension indicating a cation exchange adsorption mechanism was occurring.

Since enzymes are proteins, the effect of clay minerals on enzyme activity has also been the object of considerable study (McLaren, 1954a,b; Kobayashi and Aomine, 1967; Ross and McNeilly, 1972; Simpson and Hughes, 1978; Makboul and Ottow, 1979a,b,c). It has been shown that the activity of the adsorbed enzyme is affected by factors such as the type of enzyme, clay mineral, type of buffer, and buffer pH. Kobayashi and Aomine (1967) reported that adsorption of enzyme protein by clay minerals seems to inhibit either binding of the substrate to the enzyme or to cause a change in the configuration of the free enzyme molecule, thus reducing its

activity. The exact nature of clay-enzyme interactions is of interest because clay minerals greatly influence both the activity and fate of the enzyme. Enzymes that are fixed generally become more resistant to microbial degradation as well as physical inactivation caused by drying and temperature changes (Skujins, 1976).

The kinetic properties of immobilized enzymes have been widely studied (for reviews of this subject see McLaren and Packer, 1970, and Katchalski et al., 1971). Immobilized enzymes often obey Michaelis-Menten kinetics, but adsorption causes changes in their kinetic parameters such as the Michaelis constant (K_m) and maximum rate of reaction (V_{max}). Estimation of these kinetic parameters is accomplished by transformation of the initial velocity data of an enzyme reaction, as affected by substrate concentration, to fit one of the three linear equations derived from the Michaelis-Menten equation, $v = V_{max} \cdot S/(K_m+S)$. The Hanes-Woolf equation plot (S/v vs. S) was applied by Tabatabai and Bremner (1971) to determine the K_m value of sulfatase activity in soils. Recently, Makboul and Ottow (1979a,b,c) used this same plot to detect changes in the K_m and V_{max} values of several enzymes, including acid and alkaline phosphatases, and urease. Dick and Tabatabai (1978) studied the kinetic parameters of soil pyrophosphatase using the three linear transformation plots; the Lineweaver-Burk equation plot (1/v vs. 1/S), the Eadie-Hofstee equation plot (v vs. v/S), and the Hanes-Woolf

equation plot (S/v Vs. S). They found that the K_m and V_{max} values calculated from the different plots were all very similar. However, a large amount of information has been accumulated in which kinetic data can be interpreted by the double reciprocal Lineweaver-Burk equation (Segal, 1975). Use of this information can aid greatly in developing kinetic models to interpret results and gain insights into how an enzyme system is influenced by inhibitors, such as clay minerals, to affect changes in the velocity of a reaction.

Although the majority of the clay-enzyme studies have been carried out using enzymes purified from various biological materials, the sources of soil enzymes are microorganisms, plant residues, and plant roots (Skujins, 1967). Rogers et al. (1940, 1942) reported the detection of phosphatase and nuclease activity in corn and tomato roots, and phosphatase activity in intact wheat roots was studied by Ridge and Romira (1971). Acid phosphatase activity was also observed in sterile corn and soybean roots by Juma (1976).

The objectives of this study, therefore, were: (1) to study the effect of clay minerals on corn root acid phosphatase and inorganic pyrophosphatase activity and (2) to develop kinetic models for interpretation of the observed results.

DESCRIPTION OF METHODS

The clay minerals used in this study were kaolinite (Peerless No. 2 kaolin from South Carolina), illite (grundite illite from Illinois Clay Products, Co., Illinois), and montmorillonite (Volclay Wyoming bentonite from American Colloid Co.). The clay mineral samples used were kindly provided by Dr. A. D. Scott, Department of Agronomy, Iowa State University. They were converted to the Na-homoionic form by suspending the clays overnight in M NaCl. The next day they were washed with several small portions (25 ml) of M NaCl (until a negative test for Ca^{2+} was obtained), 50 ml of 80% ethanol, and finally with 25 ml of 95% ethanol. The clays were dried at room temperature and ground to pass a 100-mesh sieve. The physicochemical properties of the Na-homoionic clay minerals are given in Table 18. Cation exchange capacity (CEC) was determined by the NH_4^+-saturation method described by Chapman (1965), specific surface area by the ethylene glycol monoethyl ether (EGME) retention method of Heilman et al. (1965) modified by omitting the organic matter removal pretreatment and Ca^{2+} saturation of samples, and by placing a beaker containing EGME with the samples in the evacuated desiccator, and pH by a glass electrode (clay:water ratio, 1:20).

The acid phosphatase and inorganic pyrophosphatase enzymes used in this study were obtained from corn roots grown under

Table 18. Physicochemical properties of Na-homoionic clay minerals

Clay type	CEC me/100 g	Specific surface area m^2/g	Specific surface charge $me/cm^2 \times 10^{-7}$	pH in water (1:20)
Kaolinite	3.2	56	0.57	6.5
Illite	18.1	301	0.60	8.9
Montmorillonite	70.3	766	0.92	9.8

sterile conditions as described by Juma (1976). Corn seeds were germinated on agar prepared by adding 0.1 g neutralized bacteriological peptone, 0.1 g yeast extract, 10 g glucose, and 15 g agar in 1 liter distilled and deionized water (all reagents were obtained from Difco Laboratories, Detroit, Michigan). The agar solution was autoclaved for 20 minutes at 121 C and 10 ml portions transferred to sterile disposable Petri dishes (100 x 15 mm). After the agar had solidified, 5 ml of sterile water were added to each dish. Before the corn seeds were placed into the Petri dishes they were sterilized by shaking them in a 1% sodium hypochlorite solution for 20 minutes. Next they were washed with sterile water and transferred to the Petri dishes (1 seed/dish) and germinated in the dark for one week at 30 C. After germination, roots which were healthy and free of microbial growth were removed,

excised just below the seed, and weighed. The roots were then immediately homogenized in water using a 25-ml tissue grinder (Kontes of Illinois, Franklin Park, Illinois), diluted to 20 mg/ml (for acid phosphatase enzyme assays) or 50 mg/ml (for pyrophosphatase enzyme assays) and frozen at -20 C. When enzyme was needed to conduct an experiment, the corn root homogenate was thawed and filtered through Kimwipe to remove any large pieces of root that were still present.

To test the effect of clay minerals on acid phosphatase activity, the clay minerals were suspended in universal buffer (Skujins et al., 1962) at pH 4.0 and 3 ml of suspension pipetted into a 50-ml Erlenmeyer flask (clay concentration varied depending on the clay type and phosphatase studied). One ml of corn root homogenate containing 20 mg corn root (on a wet weight basis) was added, the samples were mixed thoroughly, and clay-corn root homogenate suspensions were allowed to react at room temperature for 30 minutes. This was followed by the addition of 1 ml of substrate, p-nitrophenyl phosphate, ranging in concentration from 0.80 to 4.0 mM, in universal buffer (pH 4.0), the contents of the flask again mixed, and the samples incubated one hour at 37 C. After incubation, the samples were removed, 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH added, and the contents were filtered through Whatman 2V folded filter paper. The amount of p-nitrophenol released was measured by using a Klett-

Summerson colorimeter fitted with a blue (No. 42) filter and referring to a standard curve prepared by using standards containing 0-50 μg p-nitrophenol. Controls were performed in a similar manner but p-nitrophenyl phosphate was added after addition of $CaCl_2$ and NaOH.

Pyrophosphatase activity, in the presence of clay minerals, was assayed by suspending clays in universal buffer (pH 6.0) and pipetting 1 ml of this suspension into a 50-ml plastic centrifuge tube. One ml of corn root homogenate containing 50 mg corn root (on a wet weight basis) was added, the contents mixed, and the mixture was allowed to react at room temperature for 30 minutes. Then 1 ml of tetrasodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$) ranging in concentration from 1.33 to 6.67 mM, in universal buffer (pH 6.0), was added, the contents again mixed, and the samples incubated for 1 hour at 37 C. After incubation, the samples were removed, 25 ml of cold (4 C) N H_2SO_4 added, the contents mixed, and filtered through Whatman 2V folded filter paper. Two ml aliquots were used to determine orthophosphate released as described by Dick and Tabatabai (1978). Controls were performed by adding the pyrophosphate solution to the assay mixture after incubation but before addition of N H_2SO_4. In the experiment described to determine the effect of clay minerals on acid phosphatase and pyrophosphatase activity, the effect of shaking the samples during the assay procedure was studied. The experiment was, therefore, repeated but this time the samples

were shaken for 1 hour in an incubator having a shaking accessory (Model G26 incubator, New Brunswick Scientific Co., New Brunswick, New Jersey). The concentrations of clay and substrate reported in this investigation are those present in the final volumes of the acid phosphatase and pyrophosphatase assay mixtures.

To test whether clay minerals may release any type of compound which inhibits corn root acid phosphatase activity, 80 mg of clay in a 50-ml centrifuge tube were extracted with 12 ml universal buffer (pH 4.0) by shaking for 15 minutes on a mechanical shaker. The samples were centrifuged at 20,000 x g (15,000 rpm) for 10 minutes and 3 ml of the supernatant were transferred into a 50-ml Erlenmeyer flask containing 1 ml of corn root homogenate (20 mg/ml). Acid phosphatase of this mixture was assayed using 1 ml of 5 m$\underline{M}$ $\underline{p}$-nitrophenyl phosphate as a substrate. For corn root pyrophosphatase, the test was made by extracting 80 mg of montmorillonite or illite and 800 mg of kaolinite with 8 ml universal buffer (pH 6.0) as described for acid phosphatase. One ml of the supernatant was transferred into a 50-ml plastic centrifuge tube containing 1 ml corn root homogenate (50 mg/ml). Pyrophosphatase of this mixture was assayed using 1 ml of 20 m$\underline{M}$ tetrasodium pyrophosphate as a substrate.

The degree of sorption of enzyme protein by clay minerals was determined by conducting the following experiment. 0, 10, or 100 mg clay were added to a 50-ml centrifuge

tube. To the clay minerals were added 20 ml universal buffer (pH 4.0) containing 20.0 mg corn root/ml and the contents were mixed. After 30 min of reaction time at room temperature (22 C), the clay suspensions were centrifuged at 3,000 x g (5,000 rpm) for 10 min and the acid phosphatase activity of 1 ml of the supernatant was assayed for 1 h at 37 C by using 1 ml of 5 mM p-nitrophenyl phosphate as a substrate. The degree of sorption of pyrophosphatase was studied by adding 0, 10, or 100 mg montmorillonite or illite and 0, 10, 100, or 1,000 mg kaolinite to a 50-ml centrifuge tube. To the clay minerals were added 20 ml universal buffer (pH 6) containing 50 mg corn root/ml and mixing the contents. After 30 min of reaction time at room temperature, the clay suspensions were centrifuged at 3,000 x g (5,000 rpm) for 10 min and pyrophosphatase activity of 1 ml of the supernatant was assayed for 1 h at 37 C by using 1 ml of 20 mM tetrasodium pyrophosphate as a substrate.

All values for the enzyme assays reported in this study are averages of duplicate determinations and are expressed as μmoles p-nitrophenol released·20 mg corn root^{-1}·h^{-1} for acid phosphatase activity and μmoles Pi released·50 mg corn root^{-1}·h^{-1} for inorganic pyrophosphatase activity.

RESULTS AND DISCUSSION

To study enzyme activity, it is important to know the pH at which the enzyme possesses its optimum activity. Figure 22 shows the effect of type of buffer and buffer pH on release of inorganic phosphate (Pi) in assay of pyrophosphatase of homogenized corn root. The buffers tested were citrate, acetate, and universal buffer, and all showed maximum activity at pH 6.0. However, citrate and acetate are weaker buffers while universal buffer has a strong buffering poise and it can also be used over a wide range of pH (3-12). In addition, Juma (1976) recommended universal buffer in assay of corn root acid phosphatase (pH 4.0), so that for uniformity of assay conditions between the two enzyme systems studied (acid phosphatase and pyrophosphatase), the universal buffer was selected for this work.

Results obtained from studying the inhibition of acid phosphatase and pyrophosphatase in the presence of montmorillonite and illite are shown in Figures 23-26 (samples were not shaken during incubation) and Figures 29-32 (samples were shaken during incubation). The results for inhibition of these enzymes by kaolinite are shown in Figures 27-28 (samples were not shaken during incubation) and Figures 33-34 (samples were shaken during incubation). Acid phosphatase and inorganic pyrophosphatase are hydrolytic enzymes, and their kinetic properties can be described by simple Michaelis-Menten kinetics

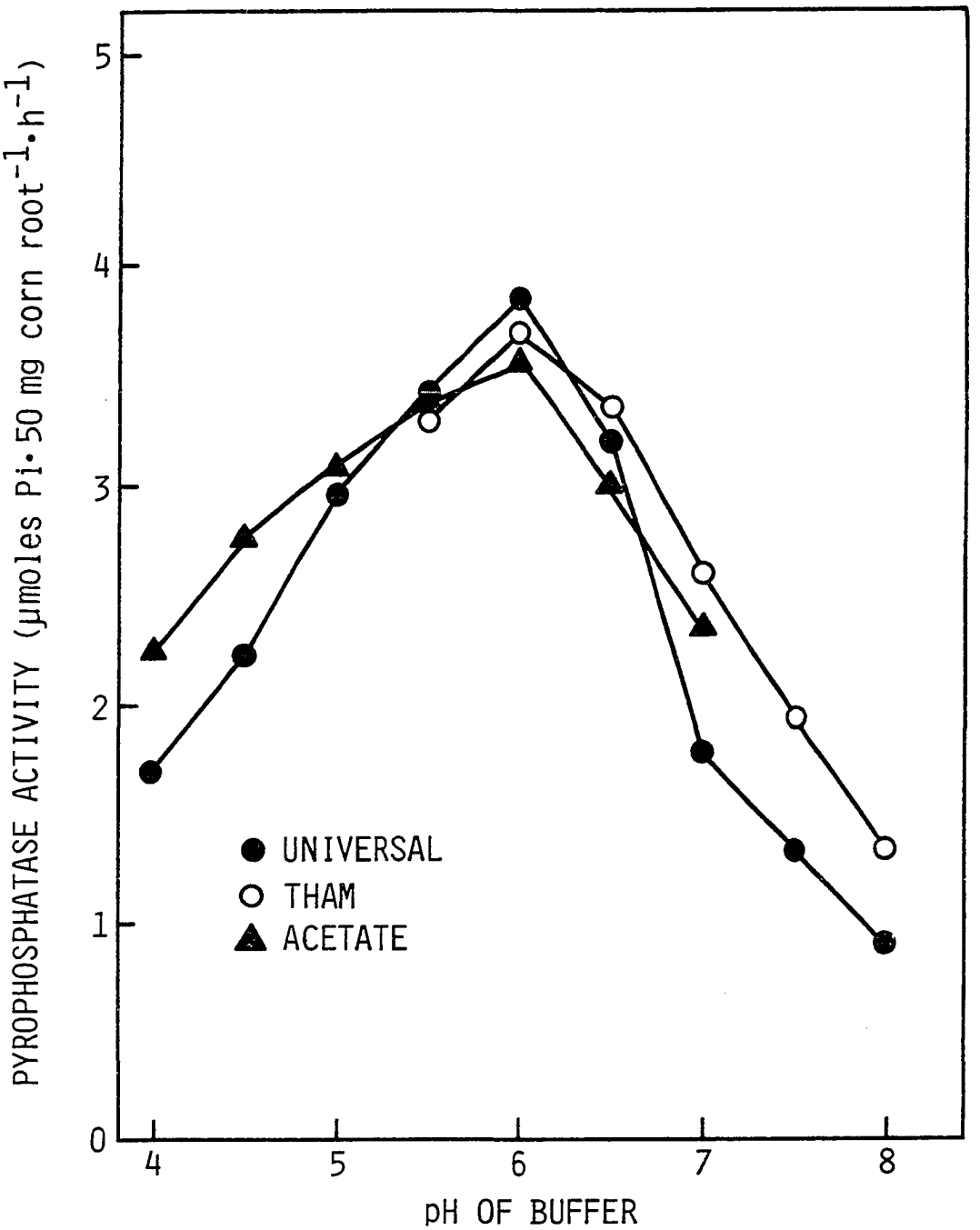


Figure 22. Effect of pH of buffer on corn root pyrophosphatase

Figure 23. Double reciprocal plot (1/v vs. 1/S) for corn root acid phosphatase activity in the presence of increasing amounts of Na-montmorillonite (samples were not shaken during incubation); velocity (v) is expressed as μmoles·p-nitrophenol released·20 mg corn root^{-1}·h^{-1} and S is in M; clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of 1/v axis intercept against clay concentration; inset B: replot of 1/Δ intercept against 1/clay concentration

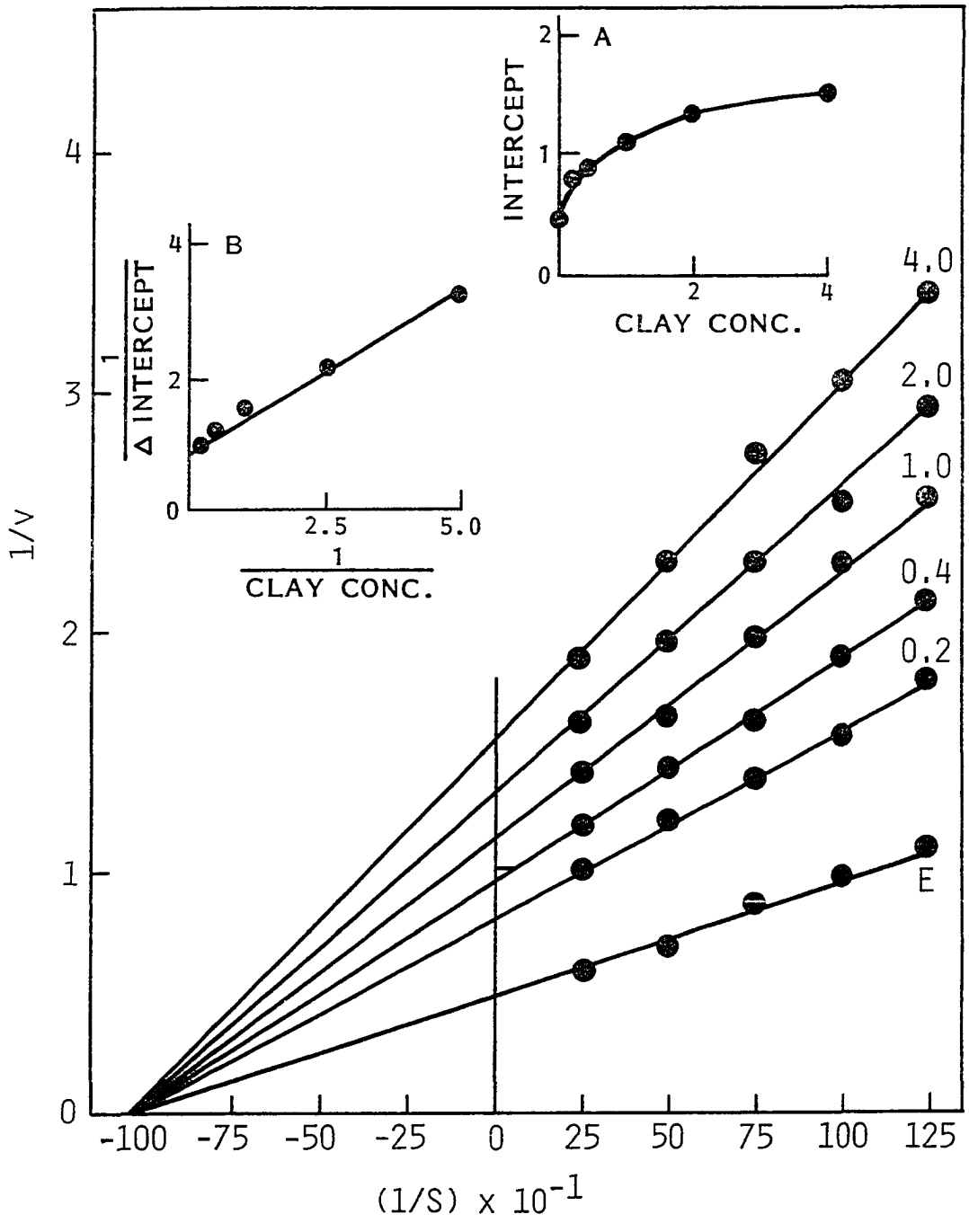

A
INTERCEPT
CLAY CONC.
B
Δ INTERCEPT
1/CLAY CONC.
4.0
2.0
1.0
0.4
0.2
E
1/V
(1/S) x 10⁻¹
-100 -75 -50 -25 0 25 50 75 100 125
0 1 2 3 4

Figure 24. Double reciprocal plot (1/v vs. 1/S) for corn root inorganic pyrophosphatase activity in the presence of increasing amounts of Na-montmorillonite (samples were not shaken during incubation); velocity (v) is expressed as μmoles Pi released·50 mg corn root^{-1}·h^{-1} and S is in M; clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of 1/v axis intercept against clay concentration; inset B: replot of 1/Δ intercept against 1/clay concentration

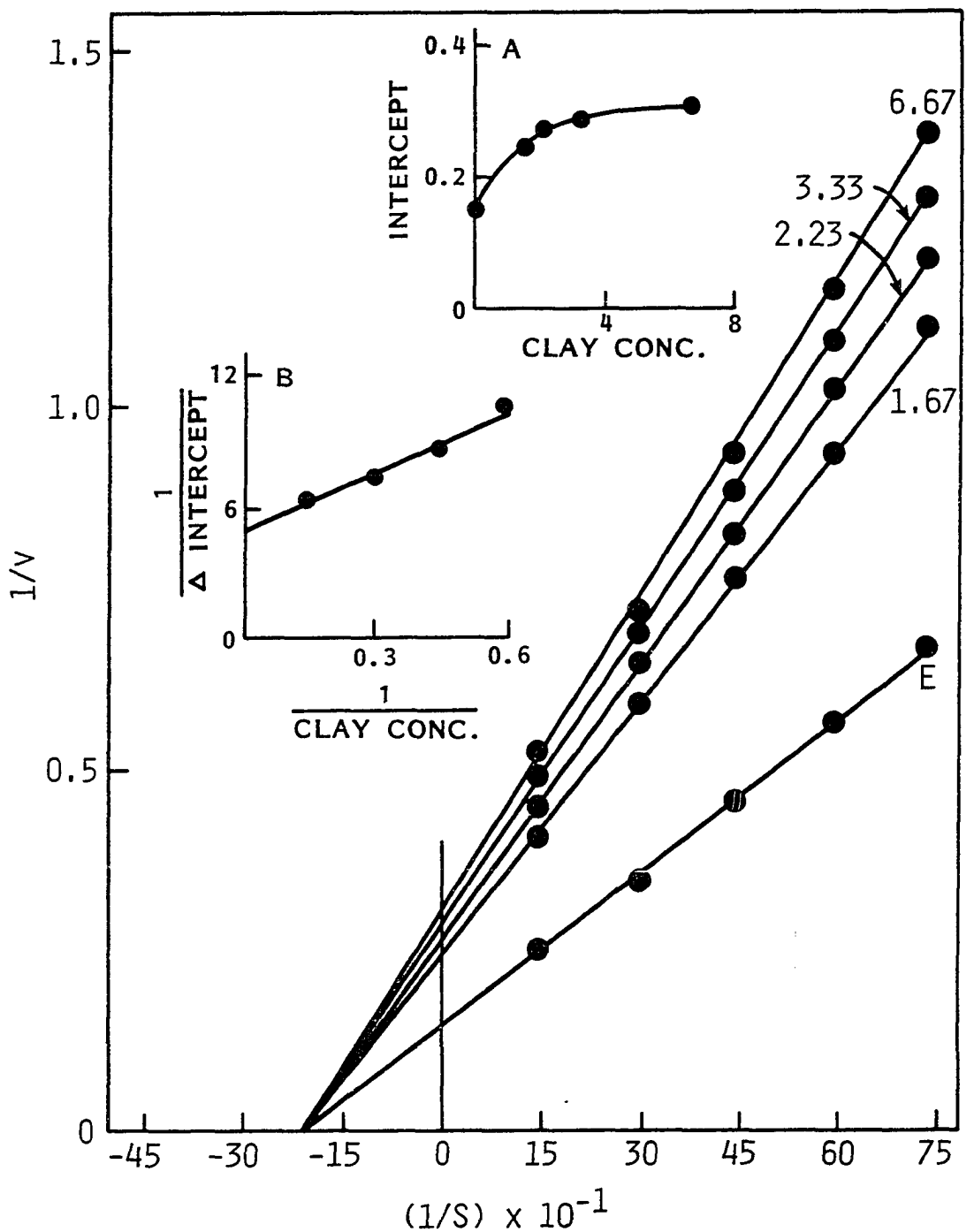

1.5
1.0
0.5
0
1/v
-45
-30
-15
0
15
30
45
60
75
(1/S) x 10^-1
6.67
3.33
2.23
1.67
E
0.4
A
0.2
0
INTERCEPT
4
8
CLAY CONC.
12
B
6
0
1
Δ INTERCEPT
0.3
0.6
1
CLAY CONC.

Figure 25. Double reciprocal plot (1/v vs. 1/S) for corn root acid phosphatase activity in the presence of increasing amounts of Na-illite (samples were not shaken during incubation); velocity (v) is expressed as μmoles p-nitrophenol released·20 mg corn root^{-1}·h^{-1} and S is in M; Clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of 1/v axis intercept against clay concentration; inset B: replot of 1/Δ intercept against 1/clay concentration

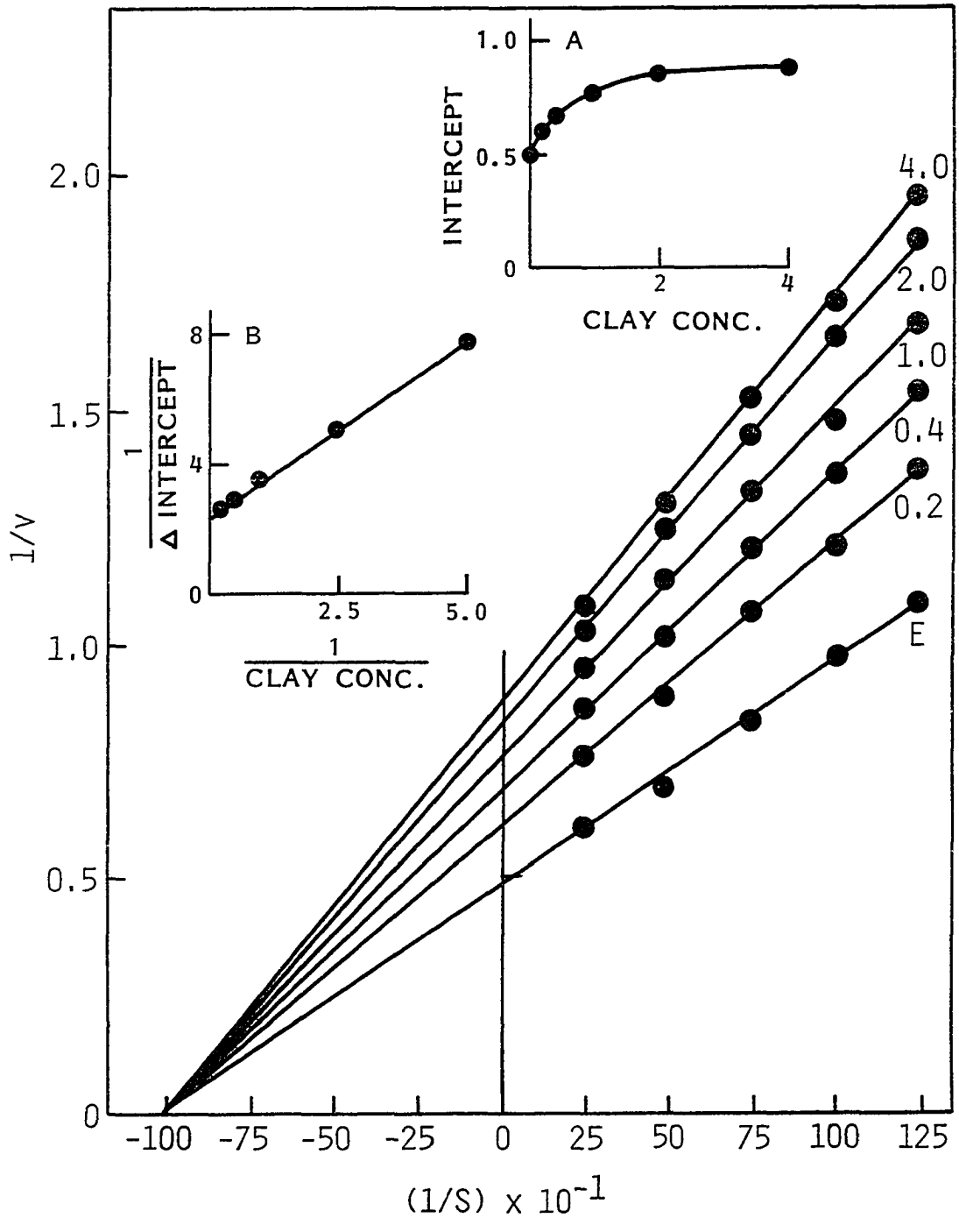

1.0
A
INTERCEPT
0.5
0
2
4
CLAY CONC.
8
B
1
Δ INTERCEPT
4
0
2.5
5.0
1
CLAY CONC.
4.0
2.0
1.0
0.4
0.2
E
2.0
1.5
1.0
0.5
0
1/V
-100
-75
-50
-25
0
25
50
75
100
125
(1/S) x 10-1

Figure 26. Double reciprocal plot (1/v vs. 1/S) for corn root inorganic pyrophosphatase activity in the presence of increasing amounts of Na-illite (samples were not shaken during incubation); velocity (v) is expressed as μmoles Pi released· 50 mg corn root^{-1}·h^{-1} and S is in M; clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of 1/v axis intercept against clay concentration; inset B: replot of 1/Δ intercept against 1/clay concentration

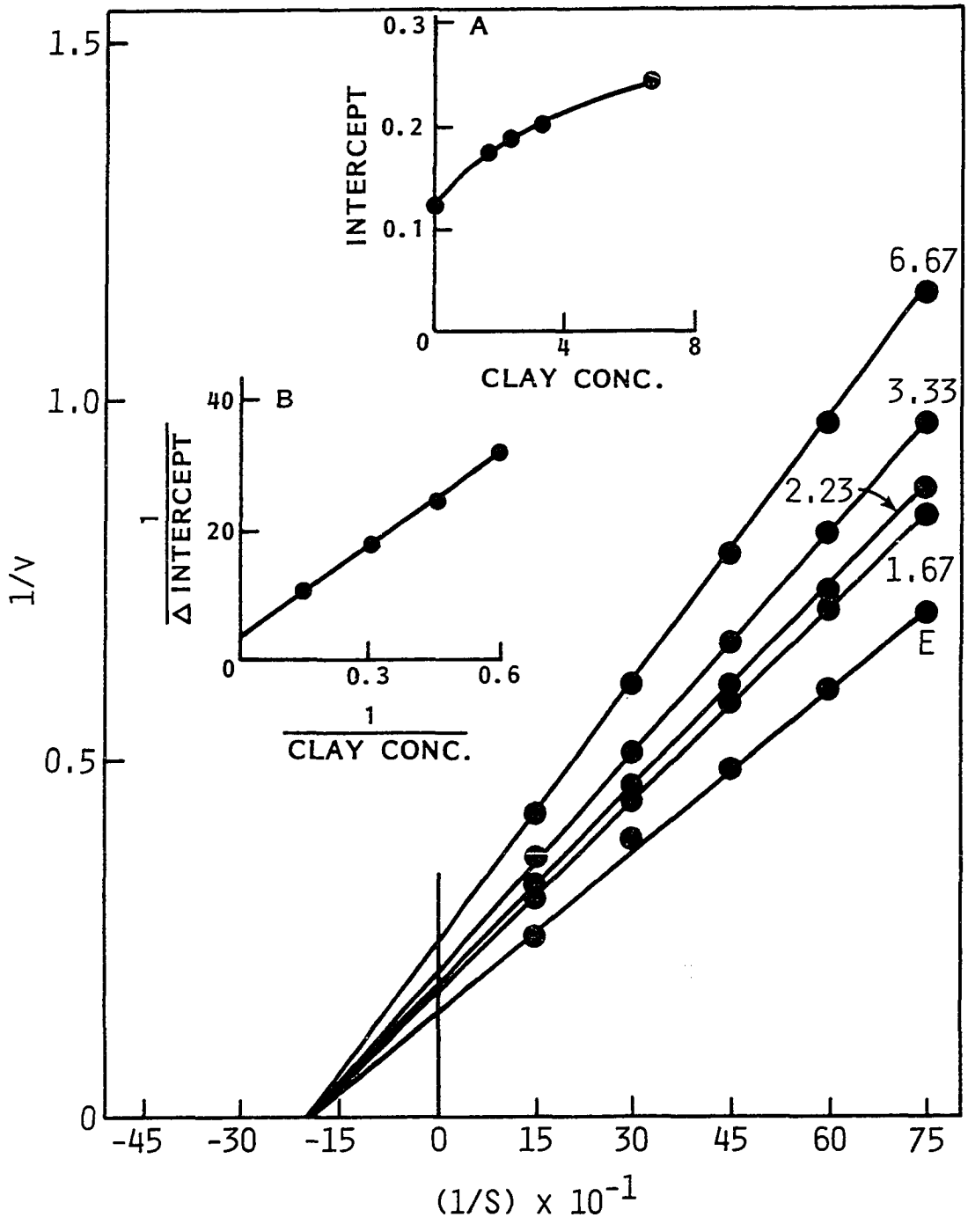

1.5
1.0
0.5
0
1/v
-45
-30
-15
0
15
30
45
60
75
(1/S) x 10^-1
6.67
3.33
2.23
1.67
E
0.3
A
0.2
0.1
INTERCEPT
0
4
8
CLAY CONC.
40
B
20
1/Δ INTERCEPT
0
0.3
0.6
1/CLAY CONC.

Figure 27. Double reciprocal plot (1/v vs. 1/S) for corn root acid phosphatase activity in the presence of increasing amounts of Na-kaolinite (samples were not shaken during incubation); velocity (v) is expressed as μmoles p-nitrophenol released· 20 mg corn root^{-1}·h^{-1} and S is in M; clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of the slope against clay concentration; inset B: replot of 1/Δ slope against 1/clay concentration

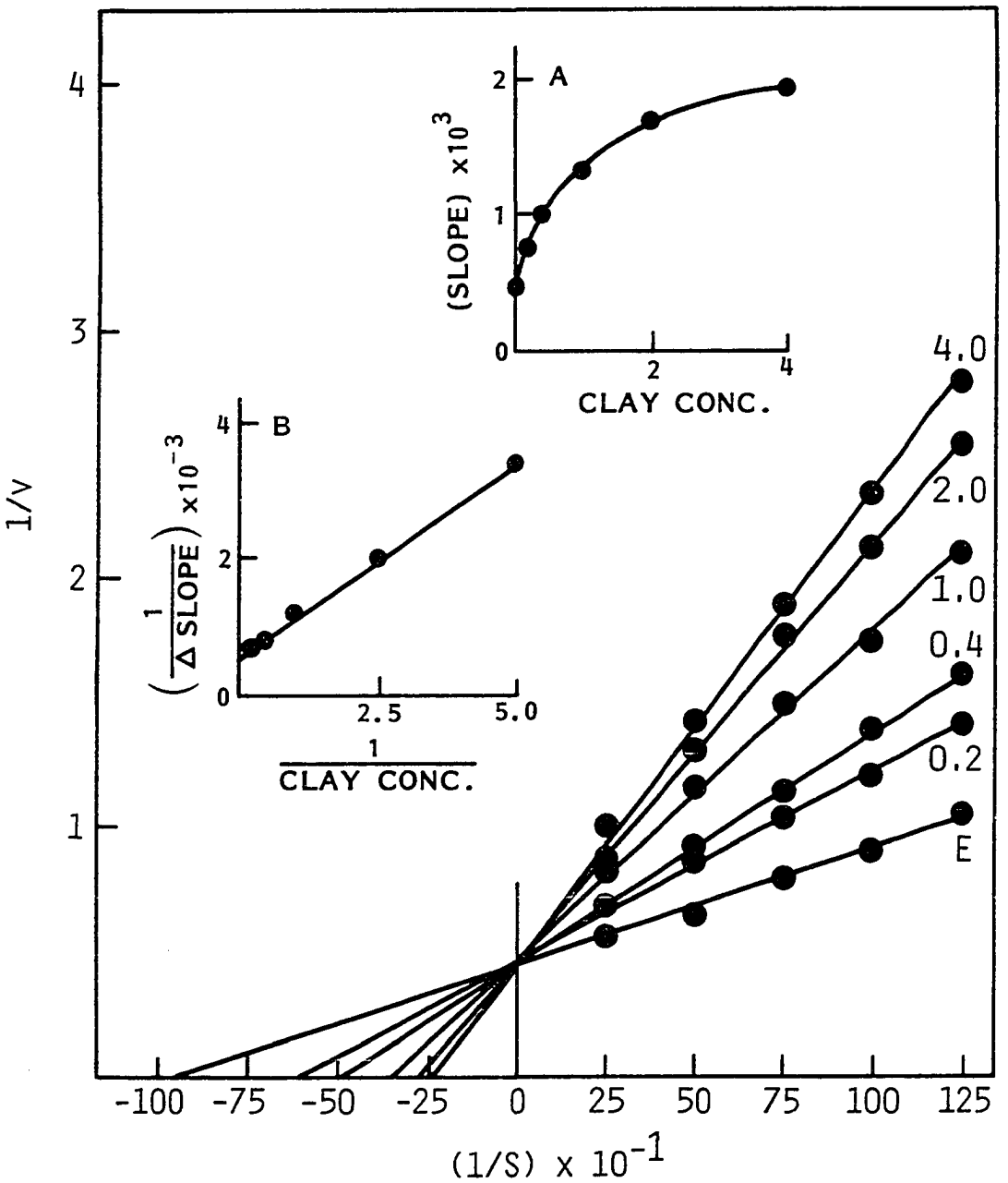

1/V
(1/S) x 10^-1
-100 -75 -50 -25 0 25 50 75 100 125
1 2 3 4
4.0
2.0
1.0
0.4
0.2
E
A
(SLOPE) x10^3
CLAY CONC.
B
(1/ΔSLOPE) x10^-3
1/CLAY CONC.
2.5
5.0

Figure 28. Double reciprocal plot (1/v vs. 1/S) for corn root inorganic pyrophosphatase activity in the presence of increasing amounts of Na-kaolinite (samples were not shaken during incubation); velocity (v) is expressed as μmoles Pi released· 50 mg corn root^{-1}·h^{-1} and S is in M; clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of the slope against clay concentration; inset B: replot of 1/Δ slope against 1/clay concentration

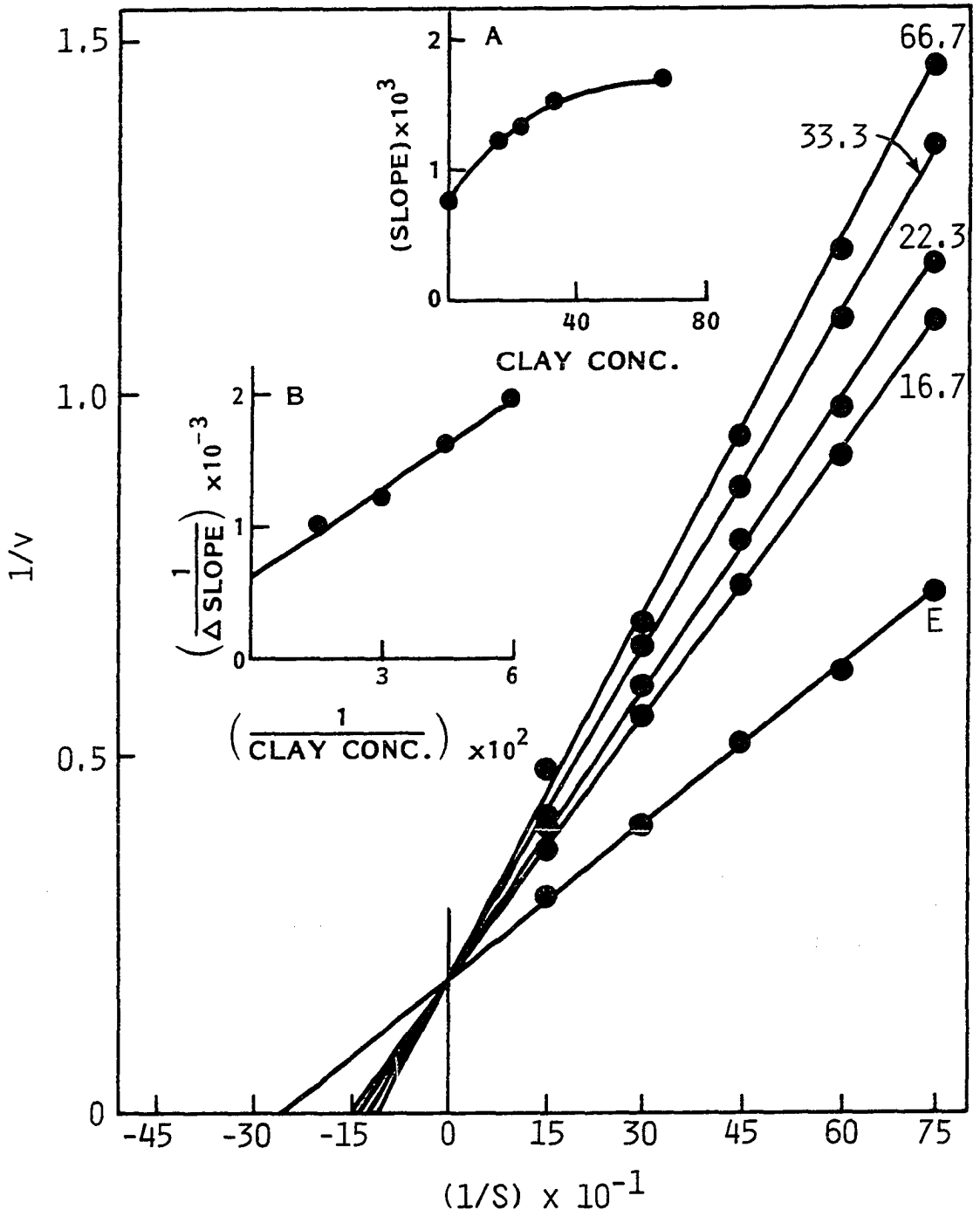

1.5
1.0
0.5
0
1/v
-45
-30
-15
0
15
30
45
60
75
(1/S) x 10^-1
66.7
33.3
22.3
16.7
E
A
(SLOPE)x10^3
CLAY CONC.
B
(1/ΔSLOPE) x10^-3
(1/CLAY CONC.) x10^2

Figure 29. Double reciprocal plot (1/v vs. 1/S) for corn root acid phosphatase activity in the presence of increasing amounts of Na-montmorillonite (samples were shaken during incubation); velocity (v) is expressed as μmoles p-nitrophenol released·20 mg corn root^{-1}·h^{-1} and S is in M; clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of 1/v axis intercept against clay concentration; inset B: replot of 1/Δ intercept against 1/clay concentration

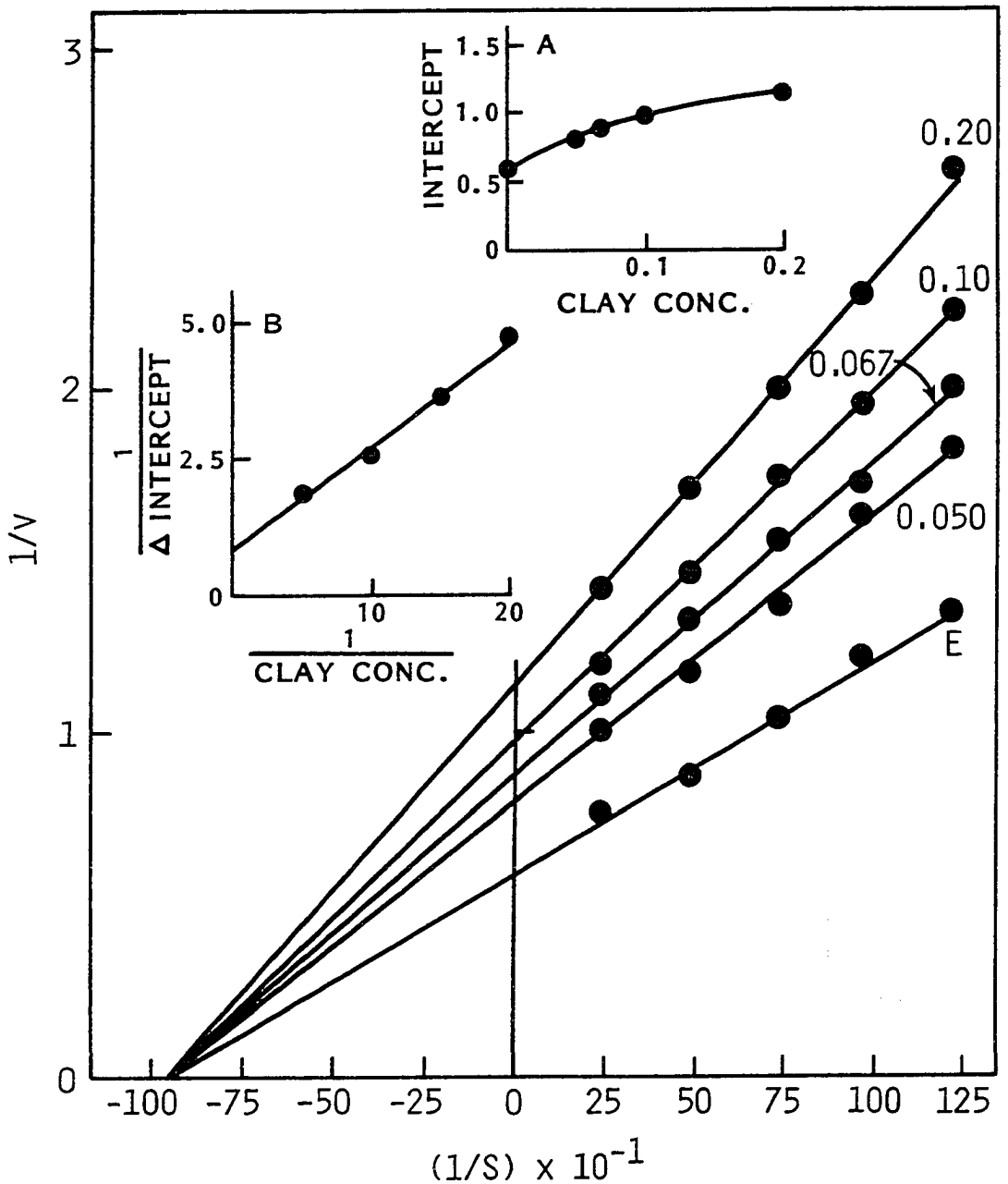

1/V
(1/S) x 10⁻¹
0.20
0.10
0.067
0.050
E
INTERCEPT
A
CLAY CONC.
Δ INTERCEPT
B
1/CLAY CONC.

Figure 30. Double reciprocal plot (1/v vs. 1/S) for corn root inorganic pyrophosphatase activity in the presence of increasing amounts of Na-montmorillonite (samples were shaken during incubation); velocity (v) is expressed as μmoles Pi released· 50 mg corn root^{-1}·h^{-1} and S is in M; clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of 1/v axis intercept against clay concentration; inset B: replot of 1/Δ intercept against 1/clay concentration

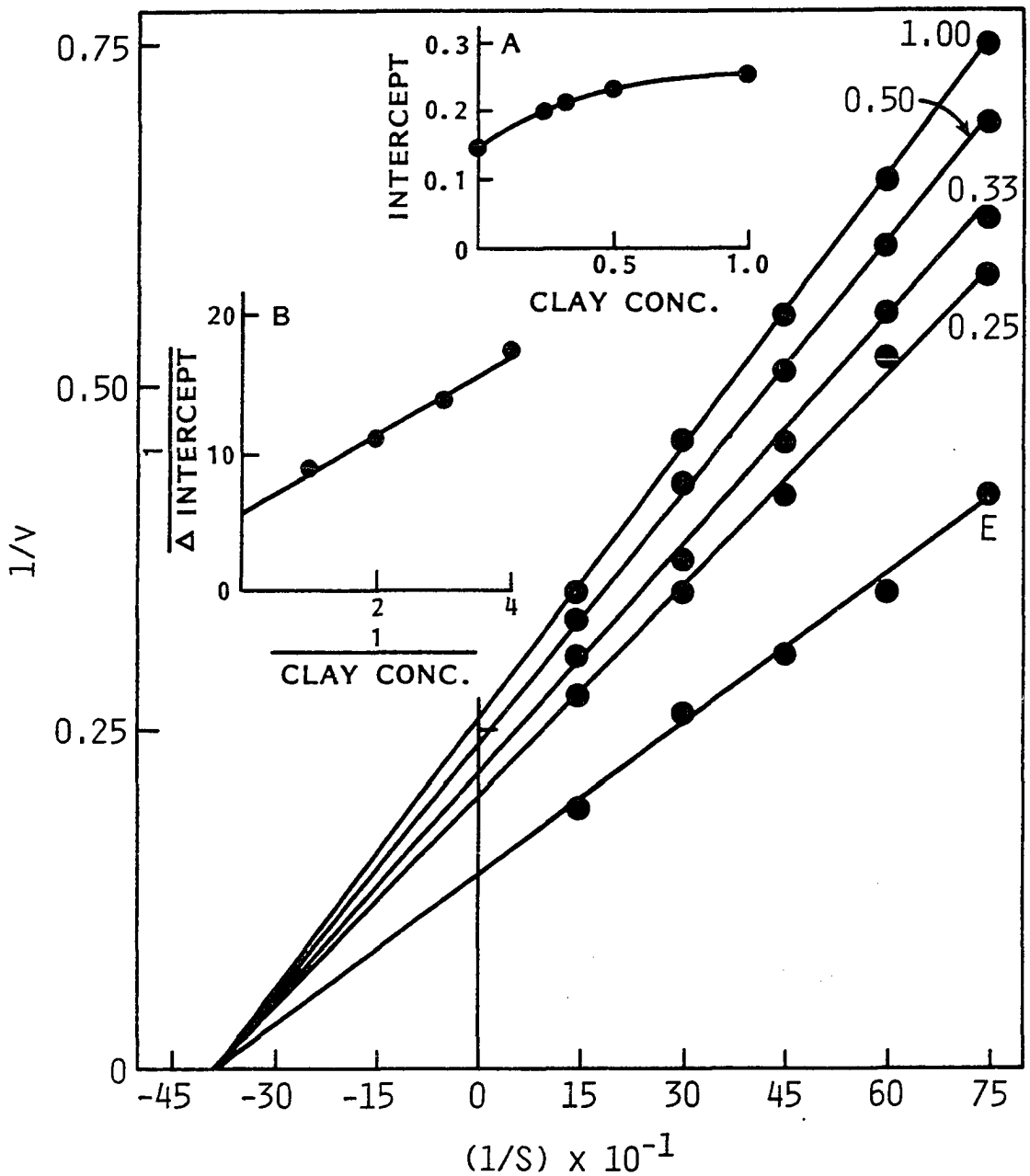

0.75
0.50
0.25
0
1/V
-45
-30
-15
0
15
30
45
60
75
(1/S) x 10⁻¹
1.00
0.50
0.33
0.25
E
A
INTERCEPT
0.3
0.2
0.1
0
0.5
1.0
CLAY CONC.
B
Δ INTERCEPT
20
10
0
2
4
1/CLAY CONC.

Figure 31. Double reciprocal plot (1/v vs. 1/S) for corn root acid phosphatase activity in the presence of increasing amounts of Na-illite (samples were shaken during incubation); velocity (v) is expressed as μmoles p-nitrophenol released·20 mg corn root^{-1}·h^{-1} and S is in M; clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of 1/v axis intercept against clay concentration; inset B: replot of 1/Δ intercept against 1/clay concentration

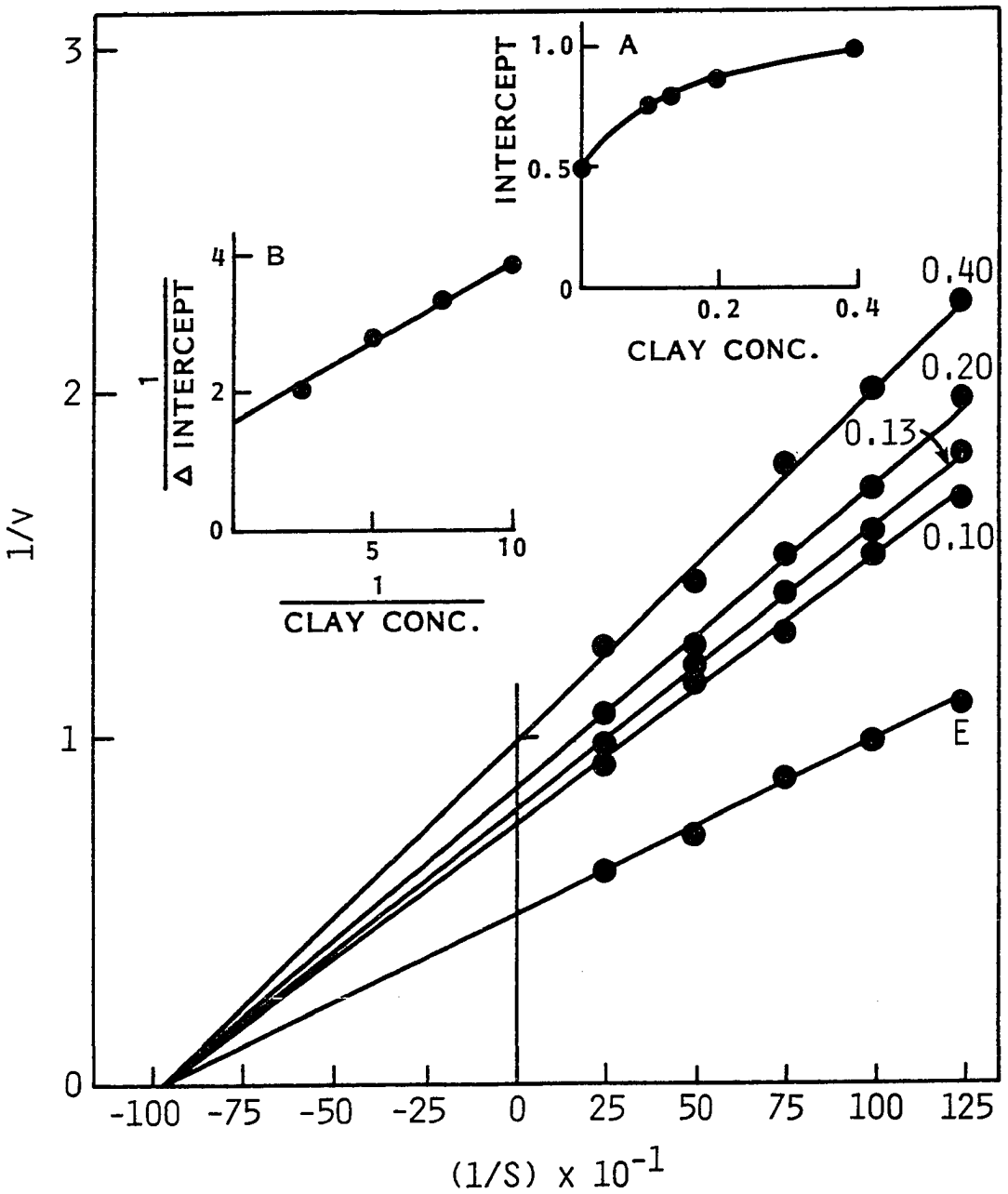

1/V
(1/S) x 10^{-1}
0.40
0.20
0.13
0.10
E
INTERCEPT
A
CLAY CONC.
Δ INTERCEPT
B
1/CLAY CONC.

Figure 32. Double reciprocal plot (1/v vs. 1/S) for corn root inorganic pyrophosphatase activity in the presence of increasing amounts of Na-illite (samples were shaken during incubation); velocity (v) is expressed as μmoles Pi released·50 mg corn root^{-1}·h^{-1} and S is in M; clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of 1/v axis intercept against clay concentration; inset B: replot of 1/Δ intercept against 1/clay concentration

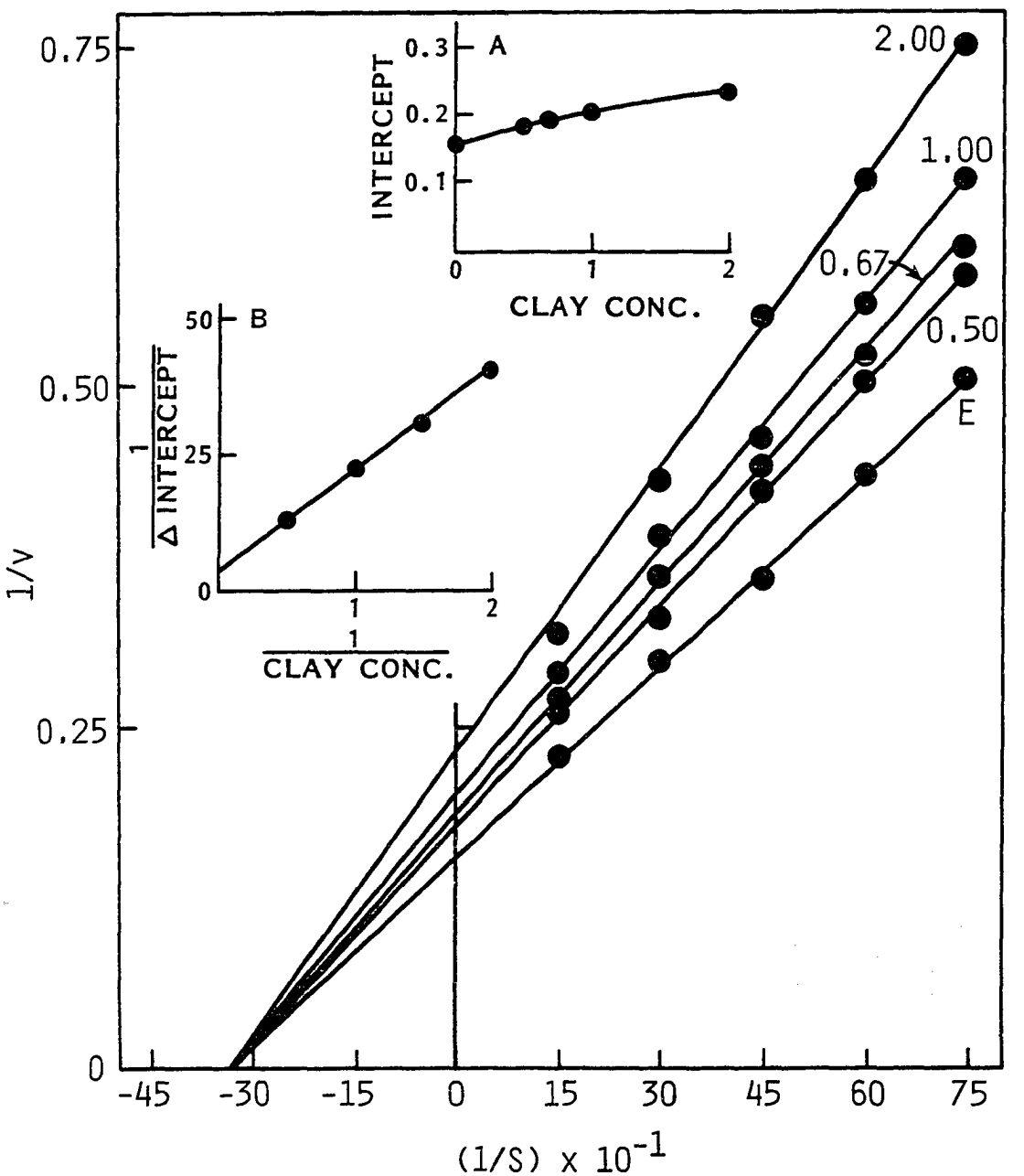

0.75
0.50
0.25
0
1/v
-45
-30
-15
0
15
30
45
60
75
(1/S) x 10^-1
2.00
1.00
0.67
0.50
E
A
INTERCEPT
0.3
0.2
0.1
0
1
2
CLAY CONC.
B
1/Δ INTERCEPT
50
25
0
1
2
1/CLAY CONC.

Figure 33. Double reciprocal plot (1/v vs. 1/S) for corn root acid phosphatase activity in the presence of increasing amounts of Na-kaolinite (samples were shaken during incubation); velocity (v) is expressed as μmoles p-nitrophenol released·20 mg corn root^{-1}·h^{-1} and S is in M; clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of the slope against clay concentration (the slopes of the linear portion of the curves in the negative 1/S region were used); inset B: replot of 1/Δ slope against 1/clay concentration

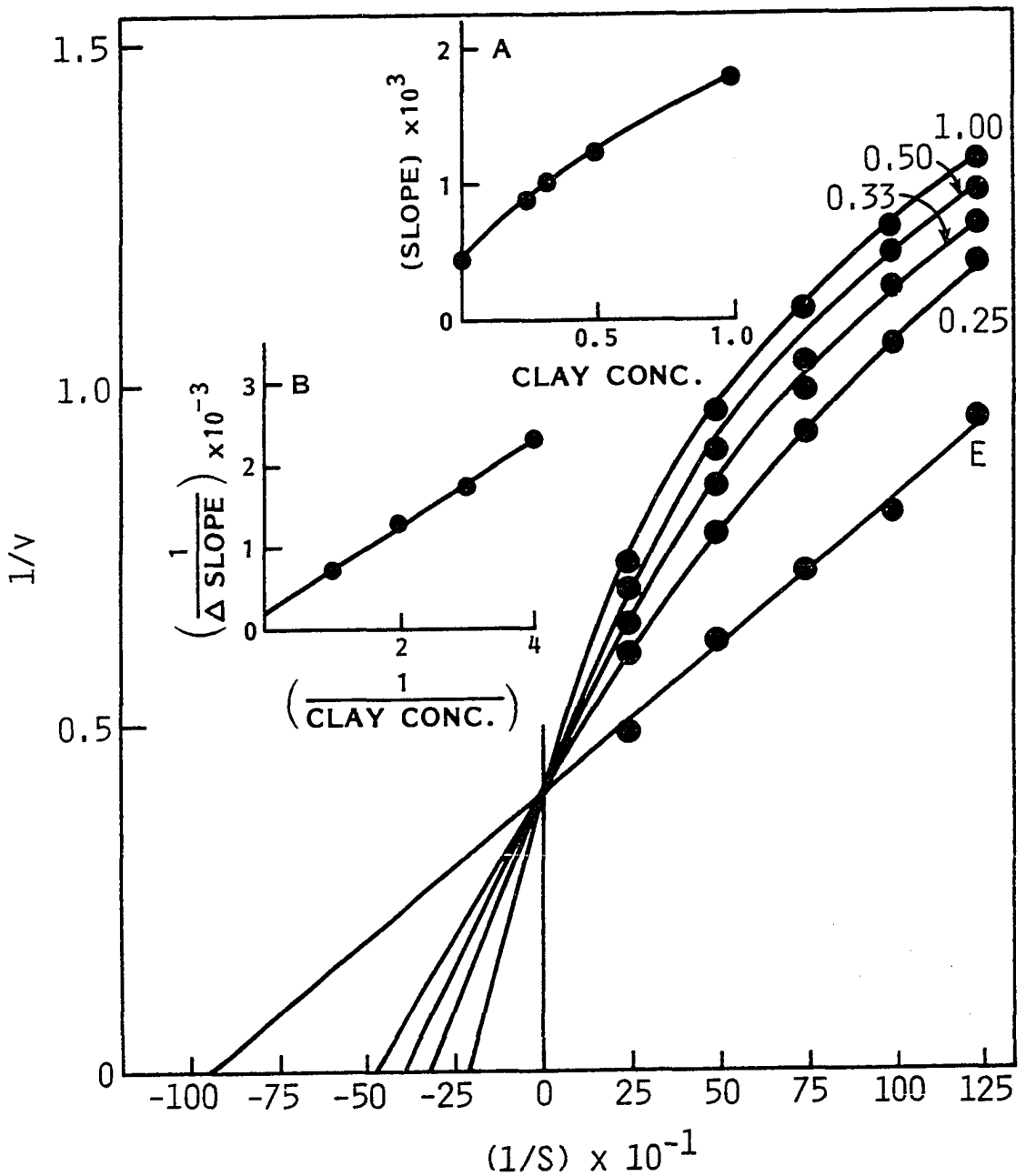
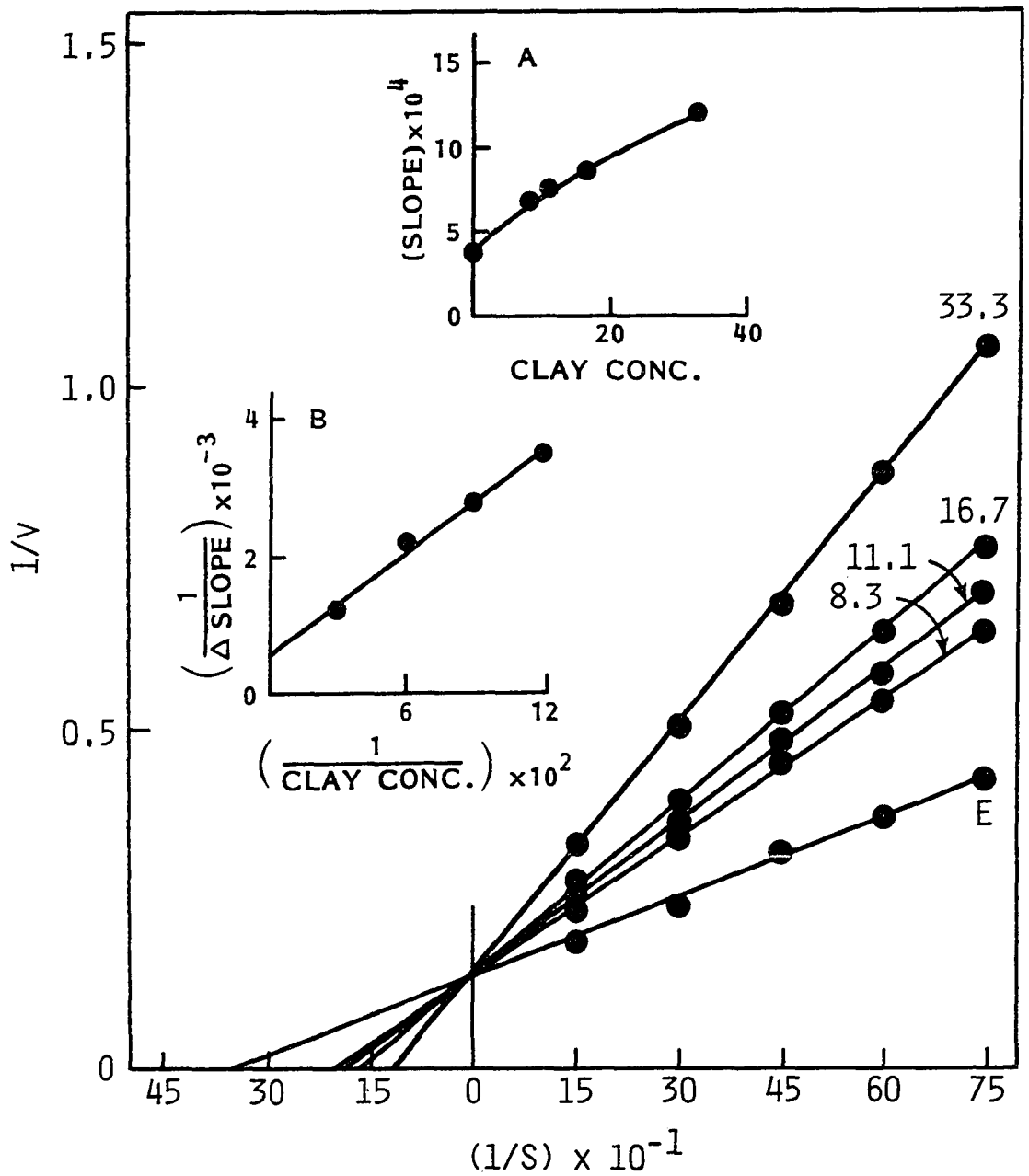

1.5
1.0
0.5
0
1/v
-100
-75
-50
-25
0
25
50
75
100
125
(1/S) x 10^-1
1.00
0.50
0.33
0.25
E
A
(SLOPE) x10^3
2
1
0
0.5
1.0
CLAY CONC.
B
(1/Δ SLOPE) x10^-3
3
2
1
0
2
4
(1/CLAY CONC.)

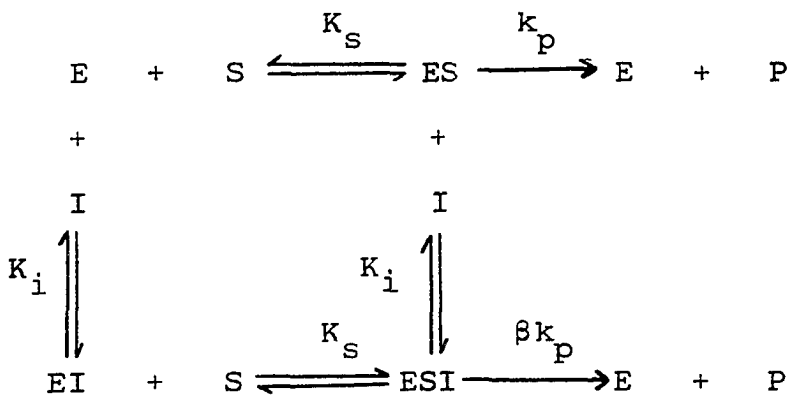
Figure 34. Double reciprocal plot (1/v vs. 1/S) for corn root inorganic pyrophosphatase activity in the presence of increasing amounts of Na-kaolinite (samples were shaken during incubation); velocity (v) is expressed as μmoles Pi released·50 mg corn root^{-1}·h^{-1} and S is in M; clay concentration is in mg/ml and E is the untreated enzyme solution (control); inset A: replot of the slope against clay concentration; inset B: replot of 1/Δ slope against 1/clay concentration


1.5
1.0
0.5
0
1/V
45
30
15
0
15
30
45
60
75
(1/S) x 10^-1
33.3
16.7
11.1
8.3
E
A
(SLOPE)x10^4
15
10
5
0
20
40
CLAY CONC.
B
(1/ΔSLOPE) x10^-3
4
2
0
6
12
(1/CLAY CONC.) x10^2

(Eivazi and Tabatabai, 1977; Dick and Tabatabai, 1978). The Michaelis-Menten equation is generally written as $v = V_{max} \cdot S/(K_s + S)$, where v is the initial enzyme reaction velocity, V_{max} is the maximum reaction velocity, S is the substrate concentration, and K_s is assumed to be the dissociation constant of the ES complex. ($K_s \approx K_m$ when the breakdown of ES to form product is much less than its breakdown to form the individual E and S molecules (Bohinski, 1976).) This equation was first proposed by Michaelis and Menten in 1913 and is based on several assumptions (Bohinski, 1976). The assumptions are: (1) a steady-state equilibrium is attained very rapidly, (2) the concentration of total enzyme (E_t) is a sum of enzyme combined with substrate (ES) and free enzyme (E_f), (3) the rate-limiting step (the slowest step) is the decomposition of enzyme-substrate complex, and (4) the maximum initial velocity (V_{max}) will be attained when the concentration of ES reaches a maximum, that is, when all the enzyme is complexed with substrate.

Inspection of Figures 23-26 and Figures 29-32 reveals that the inhibition of acid phosphatase and pyrophosphatase by montmorillonite and illite clay can be described as being partial noncompetitive in nature (Segal, 1975). This type of inhibition differs from simple noncompetitive inhibition in that the enzyme-inhibitor (EI) complex is not a dead-end complex and that the enzyme-substrate-inhibitor complex (ESI) is not a nonproductive complex. Instead, in partial noncom-

petitive inhibition, the ESI can still react to produce product, but at an impaired rate. Partial noncompetitive inhibition occurs in a system in which (1) the substrate and inhibitor combine independently to form ES, EI, and ESI complexes, and (2) the ESI can produce product, but not as effectively as ES (Segal, 1975). The equilibria describing this type of inhibition are shown below.



The various equilibria are:

$$K_s = \frac{(E)(S)}{(ES)} \quad \text{or} \quad K_s = \frac{(EI)(S)}{(ESI)}, \quad K_i = \frac{(E)(I)}{(EI)} \quad \text{or}$$

$$K_i = \frac{(ES)(I)}{(ESI)}$$

k_p = rate constant for the breakdown of ES to P, and

βk_p = rate constant for the breakdown of ESI to P ($\beta<1$).

At any inhibitor concentration, the overall velocity of product produced is $k_p(ES) + \beta k_p(ESI)$, where $\beta<1$ (the constant β is the factor by which V_{max} changes when I occupies the enzyme). Any time inhibitor is present in the system, a

portion of the enzyme exists as the less productive ESI complex, and consequently, the V_{max} value will decrease. The K_s value remains the same because the E and EI forms of the enzyme, both have equal affinity for S. The velocity equation, in the presence of inhibitor, for partial noncompetitive inhibition can be derived as follows, if we assume equilibrium conditions prevail (Segal, 1975). The overall initial velocity equation is:

$$v = k_p (ES) + \beta k_p (ESI) \tag{1}$$

Dividing the left-hand term of the velocity-dependent equation by (E_t) and the right-hand term by (E_t) expressed as the sum of all the enzyme species we obtain

$$\frac{v}{(E_t)} = \frac{k_p(ES) + \beta k_p(ESI)}{(E_f) + (ES) + (EI) + (ESI)} \tag{2}$$

Substition of the various equilibria for the partial noncompetitive model for the terms on the right-hand side of the equation containing E in them gives

$$\frac{v}{(E_t)} = \frac{k_p \frac{(S)}{k_s} + \beta k_p \frac{(S)(I)}{K_s K_i}}{1 + \frac{(S)}{K_s} + \frac{(I)}{K_i} + \frac{(S)(I)}{K_s K_i}} \quad . \tag{3}$$

Multiplying both sides of the equation by (E_t) and setting $k_p(E_t) = V_{max}$, we arrive at a velocity equation which applies to what occurs in the presence of inhibitor

$$v = \frac{V_{max}\frac{(S)}{k_s} + \beta V_{max}\frac{(S)(I)}{K_s K_i}}{1 + \frac{(S)}{k_s} + \frac{(I)}{k_i} + \frac{(S)(I)}{K_s K_i}} \qquad (4)$$

The reciprocal form of the velocity equation for partial noncompetitive inhibition is:

$$\frac{1}{v} = \frac{K_s}{V_{max}} \frac{\left(1+\frac{(I)}{K_i}\right)}{\left(1+\frac{\beta(I)}{K_i}\right)} \frac{1}{(S)} + \frac{1}{V_{max}} \frac{\left(1+\frac{(I)}{K_i}\right)}{\left(1+\frac{\beta(I)}{K_i}\right)} \quad . \qquad (5)$$

As the inhibitor concentration increases, both the slope and the intercept of the double-reciprocal (1/v vs. 1/S) plot will change, increasing by the factors in parentheses in equation 5. The curves observed will pivot counterclockwise around the point of intersection on the control curve, or at the x-axis intercept. This partial noncompetitive model is demonstrated in Figures 23-26 and Figures 29-32 for acid phosphatase and pyrophosphatase when assayed in the presence of montmorillonite and illite. Addition of these clays to the enzyme assay systems did not change the apparent K_m value but decreased the V_{max} value (Tables 19 and 20). Also, as we increase the clay concentrations, the relative amount of inhibition decreases; that is, doubling the clay concentration in the assay system, does not double the amount of inhibition observed. At infinite clay concentrations, the change in V_{max} values reaches a limit as all the enzyme is driven to form an ESI complex, which can produce product, but at a limited rate,

Table 19. Effect of clay minerals on K_m and V_{max} values of corn root acid phosphatase

Clay type	Assay condition[a]	Clay conc. (mg/ml)	K_m[b]	V_{max}[c]
Montmorillonite	NS	0	0.96	2.00
		0.20	0.96	1.24
		0.40	0.96	1.05
		1.00	0.96	0.88
		2.00	0.96	0.75
		4.00	0.96	0.65
	S	0	1.04	1.69
		0.050	1.04	1.25
		0.067	1.04	1.15
		0.10	1.04	1.04
		0.20	1.04	0.89
Illite	NS	0	1.04	2.05
		0.2	1.04	1.62
		0.4	1.04	1.44
		1.0	1.04	1.31
		2.0	1.04	1.20
		4.0	1.04	1.14
	S	0	1.03	2.04
		0.10	1.03	1.33
		0.13	1.03	1.27
		0.20	1.03	1.18
		0.40	1.03	1.02

[a]NS, samples not shaken during assay procedure; S, samples shaken during assay procedure.

[b]Expressed in m$\underline{M}$.

[c]Expressed in μmoles $\underline{p}$-nitrophenol released·20 mg corn root^{-1}·h^{-1}.

Table 19. (Continued)

Clay type	Assay condition	Clay conc. (mg/ml)	K_m	V_{max}
Kaolinite	NS	0	1.06	2.27
		0.20	1.78	2.27
		0.40	2.27	2.27
		1.00	3.00	2.27
		2.00	3.81	2.27
		4.00	4.34	2.27
	S	0	1.05	2.50
		0.25	2.12	2.50
		0.33	2.54	2.50
		0.50	3.06	2.50
		1.00	4.68	2.50

and equation 5 reduces to:

$$v = \beta V_{max} \cdot S/(K_s + S) \quad . \qquad (6)$$

Figures 27-28 and Figures 33-34 show that inhibition of acid phosphatase and pyrophosphatase by kaolinite clay follows a different mechanism than that just described. The type of inhibition observed for these enzymes in the presence of kaolinite is called partial competitive inhibition (Segal, 1975). This occurs in a system where (1) the substrate and inhibitor bind to the enzyme at different sites to yield ES, EI and ESI complexes, (2) the substrate binds to free enzyme with a greater affinity than to the EI complex, and (3) the ES and ESI complexes both yield product with equal facility. The equilibria describing partial competitive inhibition are:

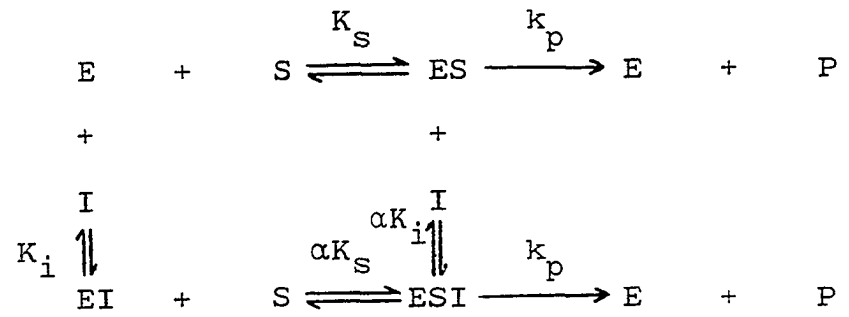
Table 20. Effect of clay minerals on K_m and V_{max} values of corn root inorganic pyrophosphatase

Clay type	Assay condition[a]	Clay conc. (mg/ml)	K_m[b]	V_{max}[c]
Montmorillonite	NS	0	4.60	6.67
		1.67	4.60	4.08
		2.23	4.60	3.73
		3.33	4.60	3.50
		6.67	4.60	3.24
	S	0	2.56	6.90
		0.25	2.56	4.95
		0.33	2.56	4.59
		0.50	2.56	4.26
		1.00	2.56	3.89
Illite	NS	0	5.32	7.09
		1.67	5.32	5.78
		2.23	5.32	5.49
		3.33	5.32	5.00
		6.67	5.32	4.15
	S	0	2.99	6.37
		0.50	2.99	5.52
		0.67	2.99	5.26
		1.00	2.99	4.95
		2.00	2.99	4.27
Kaolinite	NS	0	3.72	5.20
		16.7	6.34	5.20
		22.3	6.92	5.20
		33.3	7.86	5.20
		66.7	8.84	5.20
	S	0	2.86	7.14
		8.3	4.88	7.14
		11.1	5.26	7.14
		16.7	5.88	7.14
		33.3	8.47	7.14

[a]NS, samples not shaken during assay procedure; S, samples shaken during assay procedure.

[b]Expressed in mM.

[c]Expressed in μmoles PI released·50 mg corn root^{-1}·h^{-1}.



The various equilibria for this model are given by:

$$K_s = \frac{(E)(S)}{(ES)}, \qquad \alpha K_s = \frac{(EI)(S)}{(ESI)}, \qquad \alpha > 1$$

$$K_i = \frac{(E)(I)}{(EI)}, \qquad \alpha K_i = \frac{(ES)(I)}{ESI}$$

k_p = rate constant for the breakdown of ES and ESI to P.

The factor α is the amount K_s changes when I occupies the enzyme. At any inhibitor concentration the K_s value measured will increase because a portion of the available enzyme exists in a form (EI) that has a decreased affinity for S ($\alpha K_s > K_s$). However, increasing the substrate concentration to infinity, at the same inhibitor concentration, will drive all the enzyme to ES and ESI complexes, and because both produce product with equal facility, the V_{max} value will remain unchanged. If we assume equilibrium conditions prevail, we can derive the velocity equation for the partial competitive inhibition model in a manner similar to that described for the partial noncompetitive inhibition model (Segal, 1975).

$$v = k_p(ES) + k_p(ESI) \tag{7}$$

$$\frac{v}{(E_t)} = \frac{k_p(ES) + k_p(ESI)}{(E_f)+(ES)+(EI)+(ESI)} \tag{8}$$

$$\frac{v}{(E_t)} = \frac{k_p \frac{(S)}{K_s} + k_p \frac{(S)(I)}{\alpha K_s K_i}}{1 + \frac{(S)}{K_s} + \frac{(I)}{K_i} + \frac{(S)(I)}{\alpha K_s K_i}} \tag{9}$$

$$v = \frac{V_{max} \frac{(S)}{K_s} + V_{max} \frac{(S)(I)}{\alpha K_s K_i}}{1 + \frac{(S)}{K_s} + \frac{(I)}{K_i} + \frac{(S)(I)}{\alpha K_s K_i}} \tag{10}$$

$$\frac{1}{v} = \frac{K_s}{V_{max}} \frac{\left(1 + \frac{(I)}{K_i}\right)}{\left(1 + \frac{(I)}{\alpha K_i}\right)} \frac{1}{(S)} + \frac{1}{V_{max}} \tag{11}$$

If we plot 1/v vs. 1/S in the presence of increasing amounts of inhibitor, the slope of the curves obtained will increase by the factor within the parentheses in equation 11, while the intercept value remains unchanged. The curves will pivot counterclockwise about the point of intersection with the control curve, or at the y-axis intercept. Figures 27-28 and Figures 33-34 demonstrate similar type curves for acid phosphatase and pyrophosphatase when assayed in the presence of kaolinite. Tables 19 and 20 show that the V_{max} values remained unchanged but the apparent K_m values increased for these enzymes when increasing amounts of kaolinite were added to the enzyme system. Also, as observed for montmorillonite and illite, in the partial noncompetitive inhibition model,

increasing the clay (kaolinite) concentration did not give a corresponding decrease in enzyme activity. At increasing concentrations of clay, all the enzyme is in ES or ESI form and equation 10 reduces to:

$$v = V_{max} \cdot S/(\alpha K_S + S). \qquad (12)$$

The enzyme is present in a modified, but functional, form in which V_{max} remains constant, but a limiting K_S value is observed.

The α, β, and K_i constants in the kinetic models can be estimated by use of appropriate techniques (Segal, 1975). If we plot the y-intercept values against the clay concentrations used in the assay systems we obtain a curve as shown in insets A of Figures 23-26 and Figures 29-32. If we plot the slope values against clay concentrations we obtain a curve as shown in insets A of Figures 27-28 and Figure 33-34. Both plots are hyperbolic and consequently the limits (which would allow α, β, and K_i to be determined) are difficult to identify. A replot of the data in insets A as $1/\Delta$ intercept vs. $1/(I)$ (insets B, Figures 23-26 and Figures 29-32) or $1/\Delta$ slope vs. $1/(I)$ (insets B, Figures 27-28 and Figures 33-34) converts the relationship between y-intercept or slope values and inhibitor concentrations to a straight line. Thus, the replot of $1/\Delta$ intercept vs. $1/(I)$ has a slope of $\alpha K_i V_{max}/(1-\beta)$, an intercept on the $1/\Delta$ intercept axis of $\beta V_{max}/(1-\beta)$, and a $1/(I)$ axis intercept value of $-\beta/\alpha K_i$. The replot of

$1/\Delta$ slope vs. $1/(I)$ has a slope of $\alpha K_i V_{max}/K_s(\alpha-\beta)$, an intercept of $1/\Delta$ slope axis of $\beta V_{max}/K_s(\alpha-\beta)$, and a $1/(I)$ axis intercept value of $-\beta/\alpha K_i$.

The kinetic constants, obtained when samples were not shaken during incubation, for the partial competitive and partial noncompetitive inhibition models for corn root acid phosphatase and pyrophosphatase inhibited by clay minerals are shown in Table 21. Montmorillonite and illite clay bind to the enzymes at a specific, modifier site distinct from the substrate binding site and cause a decrease in the catalytic effectiveness of the enzymes without affecting the binding of substrate. The β constant is an indication of the degree in which the catalytic function of acid phosphatase and pyrophosphatase is impaired when bound to montmorillonite and illite. A β constant value of 1 indicates that no decrease in catalytic efficiency has occurred, but a β constant value less than 1 indicates a decrease in the catalytic efficiency of an enzyme. Table 21 shows that the β constant value for acid phosphatase and pyrophosphatase bound to kaolinite is 1, but that the β constant values for acid phosphatase bound to montmorillonite and illite are 0.33 and 0.53, respectively. For pyrophosphatase, the β constant value determined for enzyme bound to illite is 0.32 which is less than that of pyrophosphatase bound to montmorillonite where the β constant value is 0.42.

Kaolinite also binds to acid phosphatase and pyrophosphatase at a site distinct from the substrate binding site, but

Table 21. Kinetic constants of corn root acid phosphatase and inorganic pyrophosphatase inhibited by clay minerals

Enzyme	Clay type	Assay condition[a]	α constant	β constant[b]	K_i (mg/ml)[b]
Acid phosphatase	Montmorillonite	NS	1.0	0.33 (0.33)	0.15 (0.16
		S	1.0	0.29 (0.34)	0.086 (0.074)
	Illite	NS	1.0	0.53 (0.53)	0.32 (0.24)
		S	1.0	0.42 (0.43)	0.068 (0.068)
	Kaolinite	NS	4.6	1.0	0.19
		S	10.9	1.0	0.19
Pyro-phosphatase	Montmorillonite	NS	1.0	0.42 (0.41)	0.74 (0.81)
		S	1.0	0.44 (0.45)	0.23 (0.22)
	Illite	NS	1.0	0.32 (0.28)	5.4 (4.8)
		S	1.0	0.39 (0.39)	1.6 (1.7)
	Kaolinite	NS	3.2	1.0	10.7
		S	5.4	1.0	8.0

[a]NS, samples were not shaken during incubation assay procedure; S, samples were shaken during assay procedure.

[b]Values in parentheses were obtained from the $1/\Delta$ intercept replot technique. The remainder of the values were obtained from the $1/\Delta$ slope replot technique.

instead of changing the catalytic effectiveness of the enzymes, it changes the affinity of the enzyme for the substrate. The α constant gives an indication of how much the binding affinity is altered when acid phosphatase and pyrophosphatase is bound to kaolinite clay. Table 21 shows that binding of substrate by acid phosphatase is affected to a greater degree by kaolinite (α = 4.6) than is pyrophophatase (α = 3.2) (samples were not shaken during incubation).

The K_i value, measured when the samples were not shaken during incubation, is an estimate of the dissociation constant of the clay-enzyme complex and can be regarded in simple terms as the affinity the enzyme has for the clay minerals (the larger the K_i value, the less the affinity for the clay). Acid phosphatase has a greater affinity for all three clay minerals studied than does pyrophosphatase (Table 21) and the K_i values determined for acid phosphatase for the three clay minerals were very similar, ranging only from 0.15 mg/ml for montmorillonite to 0.19 mg/ml for kaolinite. For pyrophosphatase, the affinity of this enzyme for montmorillonite was greater (K_i = 0.74 mg/ml) than for illite (K_i = 5.4 mg/ml) which is greater than that observed for kaolinite (K_i = 10.7 mg/ml).

Table 21 and Figures 29-32 show the effect of shaking the assay mixture during the 1 h incubation period used in the assay procedure on acid phosphatase and pyrophosphatase activity. Shaking the samples during incubation was included

as part of this study because enzymes that are bound to solid supports exhibit activity that is greatly affected by diffusion properties. In the immediate vicinity of the enzyme catalytic site, the "concentrations" of substrate and product may be much different than that in the bulk solution. This is because immobilized enzyme particles in aqueous suspension are surrounded by an unstirred layer of solvent. The thickness of this layer is determined by the rate of agitation of the assay mixture. In the course of the immobilized enzyme reaction, a concentration gradient of substrate is established across the unstirred layer (Katchalski et al., 1971). Figures 29-32 show that the type of inhibition observed for acid phosphatase and pyrophosphatase by clay minerals remains unchanged when the samples were shaken to decrease these adverse effects. However, some variation in the kinetic constants occurred. Shaking the samples caused an increase in the α constant for acid phosphatase and pyrophosphatase bound to kaolinite from 4.6 to 10.9 and from 3.2 to 5.4, respectively. This suggests that the affinity of substrate by the enzyme was less when samples were not shaken than that measured when samples were shaken. The β constants for acid phosphatase and pyrophosphatase bound to montmorillonite and illite remain relatively unchanged. Since this is the constant which indicates the effect that clays have on the enzyme catalytic efficiency, diffusional effects on this aspect of the overall enzyme mechanism probably are not too important.

The K_i values for montmorillonite and illite decreased greatly when samples were shaken. For acid phosphatase bound to montmorillonite, the K_i values decreased from 0.15 to 0.086 mg/ml, while the K_i constant for acid phosphatase bound to illite decreased from 0.32 to 0.068 mg/ml. No change in the K_i constant for acid phosphatase bound to kaolinite was observed, however. For pyrophosphatase, a similar observation was made. The K_i constant of this enzyme bound to montmorillonite decreased from 0.74 to 0.23 mg/ml and for illite it decreased from 5.4 to 1.6 mg/ml. A slight decrease in the K_i constant for pyrophosphatase bound to kaolinite occurred when samples were shaken during incubation, with the K_i values changing from 10.7 to 8.0 mg/ml.

Although the K_i values for acid phosphatase bound to kaolinite did not change when samples were shaken during incubation, shaking did cause an effect. Figure 33 indicates that when acid phosphatase was assayed in the presence of kaolinite, and the samples were shaken during incubation, the curves obtained for the double-reciprocal plot were not entirely linear but curved downward at the lower substrate concentration region. This indicates that acid phosphatase was not inhibited to the degree predicted by the partial competitive model at low concentrations of substrate, but did conform at high concentrations. Proteins adsorbed to charged surfaces are greatly influenced by pH, ionic strength, temperature, and addition of substrate. Variations in any

of these parameters may lead to partial or total desorption of the protein from the surface to which it is bound (Katchalski et al., 1971). Addition of substrate to the assay mixture containing corn root acid phosphatase and kaolinite may have caused a partial desorption of the enzyme from the clay. The subsequent increased activity observed above that predicted by the partial competitive model could, therefore, result. This desorption phenomenon occurred to a much greater degree when the samples were shaken during incubation than when they were not shaken.

The inhibition of acid phosphatase and pyrophosphatase by clay minerals has been assumed, to this point, to be due to binding of the enzyme to the clay minerals. Feigenbaum and Shainberg (1975), however, demonstrated that Fe and Al can be released from clay minerals and these metals, as well as others, have been shown to be potent inhibitors of acid phosphatase (Juma and Tabatabai, 1977) and pyrophosphatase (Naganna and Menon, 1948). The possibility that inhibition of acid phosphatase and pyrophosphatase was due to dissolution of metal ions from clay minerals was tested by assaying corn root homogenate for these enzymes treated with clay extracts. Table 22 shows that the clay extracts had no effect on activity, indicating that clay minerals themselves caused the inhibition, due to a physico-chemical interaction occurring in the inhibition mechanism. Further support for this hypothesis is shown in Table 23 where the activity of

Table 22. Effect of clay extracts on acid phosphatase and pyrophosphatase activity

Enzyme	Clay extracted	Activity[a]
Acid phosphatase	None	1.01
	Kaolinite	1.04
	Montmorillonite	1.02
	Illite	1.01
Pyrophosphatase	None	4.00
	Kaolinite	3.94
	Montmorillonite	3.95
	Illite	4.02

[a]Acid phosphatase activity is expressed as μmoles p-nitrophenol released·20 mg corn root^{-1}·h^{-1} and pyrophosphatase activity is expressed as μmoles Pi released·50 mg corn root^{-1}·h^{-1}.

acid phosphatase was measured after the corn root homogenate was allowed to react for 30 min with the clay minerals. The clay minerals were then centrifuged out of the assay mixture and an aliquot of the supernatant assayed for acid phosphatase and pyrophosphatase activity. It was found that most of the activity remained with the clay minerals and only a small portion was present in the supernatant. When 100 mg clay were added to 20 ml universal buffer (pH 4) containing 400 mg of homogenized corn root, 97-99% of the acid phosphatase activity

Table 23. Activity of acid phosphatase and pyrophosphatase after sorption onto clay minerals

Enzyme	Clay added (mg)	Residual activity after sorption by clay specified[a]		
		Kaolinite	Montmorillonite	Illite
Acid phosphatase	0	0.97	0.97	0.97
	10	0.086 (91)	0.086 (91)	0.069 (93)
	100	0.024 (97)	0.012 (99)	0.019 (98)
Pyro-phosphatase	0	3.99	3.99	3.99
	10	3.13 (22)	2.04 (49)	2.89 (28)
	100	2.88 (28)	0.36 (91)	1.52 (62)
	1000	0.68 (83)	-	-

[a]Acid phosphatase activity is expressed as μmoles p-nitrophenol released·20 mg corn root^{-1}·h^{-1} and pyrophosphatase activity is expressed as μmoles Pi released·50 mg corn root^{-1}·h^{-1}. Values in parentheses are the percentages of inhibition caused by clay sorption of corn root enzyme.

was sorbed onto the clay minerals, regardless of the type of clay mineral added. When 100 mg clay was added to 20 ml universal buffer containing 1000 mg of homogenized corn root, 91% of the activity was sorbed onto montmorillonite, 62% onto illite, and 28% onto kaolinite (Table 23).

The binding of enzymes to solid supports, such as clay minerals, can occur by several different mechanisms. The most dominant mechanisms have been described by Weetall (1975). These include (1) adsorption, (2) entrapment, (3) microen-

capsulation, (4) ion exchange, (5) cross-linking, (6) adsorption and cross-linking, (7) copolymerization, and (8) covalent attachment. All of these mechanisms have been suggested to explain the immobilization and protection of enzymes in the soil environment (Burns, 1978). The activity of bound enzymes, however, is generally lower than the corresponding soluble enzymes. This lower activity is due to reaction of amino acid side chains necessary for catalysis or substrate binding, steric hindrance of the approach of substrate, disruption of the three-dimensional structure of the protein, and diffusional limitations (Royer, 1975). Decreases in activity which occur upon immobilization can result in (1) an increase in the K_m value or (2) a decrease in the V_{max} value. Kaolinite inhibition of acid phosphatase and pyrophosphatase is an example where a change in the K_m value occurred and is considered to be due to the alteration of the binding site of the enzymes. Steric hindrance could be an important factor in causing inhibition of acid phosphatase and pyrophosphatase activity if these enzymes bind to the kaolinite clay surface in such a way as to orient the substrate binding site towards the clay surface. This could result in blocking of substrate molecules from interacting with the enzymes. In addition, electrostatic effects may play an important role in the action of immobilized enzymes. Changes in the apparent K_m observed upon inhibition of acid phosphatase and pyrophosphatase by kaolinite could be related to the unequal distribution

of substrate between the charged clay support and the surrounding solution. If the products of an enzyme reaction are attracted to the support, with the exclusion of substrate, a concentration gradient of substrate would be established. Saturation of enzyme would, therefore, occur at higher substrate concentration levels than for enzyme free in solution. This would increase the apparent K_m value. For acid phosphatase and pyrophosphatase inhibition by kaolinite, the combination of the steric effect and the electrostatic effect in influencing activity may be important in establishing the type of inhibition mechanism observed.

Another possibility why the kinetic model found for the inhibition of acid phosphatase and pyrophosphatase by kaolinite differed from that of montmorillonite and illite is due to the microenvironment in which the enzyme performs catalysis. This microenvironment, in which the bound enzyme functions, can be influenced by the physico-chemical characteristics of the enzyme support and can also be changed as a result of the enzyme activity itself (Katchalski et al., 1971). Stout (1939) found that finely ground kaolinite can adsorb larger amounts of phosphate than can montmorillonite. Since phosphate is a product of both the acid phosphatase and pyrophosphatase reactions, an increase in its concentration would occur on the kaolinite surface relative to that of montmorillonite. A greater concentration of phosphate in the vicinity of the substrate binding site of the enzymes on the kaolinite

surface would allow for greater opportunity of the phosphate to act as an inhibitor of acid phosphatase and pyrophosphatase. Browman and Tabatabai (1978) and Juma and Tabatabai (1978) have shown phosphate to be a competitive inhibitor of acid phosphatase and Josse and Wong (1971) have shown phosphate to be a competitive inhibitor of pyrophosphatase. Inhibition of these enzymes upon increasing concentrations of kaolinite in the assay mixture would lead to all of the enzyme being bound to the clay. However, the initial velocity of the acid phosphatase and pyrophosphatase bound to kaolinite would always be greater than zero, providing the Pi concentrations did not quickly reach levels that would exclude binding of substrate. This is consistent with the experimental results obtained in the work reported, where inhibition of acid phosphatase and pyrophosphatase reached a limiting value other than zero.

Although the mechanism of binding acid phosphatase and pyrophosphatase to montmorillonite and illite may be similar to that of kaolinite, the exact nature of the binding to montmorillonite and illite must be different than that of kaolinite. This is because instead of a change in K_m occurring, a change in V_{max} was observed. Upon increasing additions of montmorillonite and illite, the V_{max} values determined for acid phosphatase and pyrophosphatase decreased. A decrease in V_{max} can result from total destruction of the bound enzyme, partial reduction of activity of the bound

enzyme, or a combination of both (Royer, 1975). A total destruction of bound enzyme would not explain the results observed for acid phosphatase and pyrophosphatase when bound to montmorillonite and illite. This is because even when saturation levels of clay concentration are approached in the assay mixture, the activity, instead of approaching zero, approached some other limiting value. This interpretation of results is contrasted with that of Hughes and Simpson (1978) when they assumed that arylsulfatase adsorbed to montmorillonite clay was either loosely held (in which activity was considered to remain 100%) or tightly bound (in which activity was considered to be zero).

It is hypothesized that acid phosphatase and pyrophosphatase are bound onto montmorillonite and illite by ionic interactions. At pH values below the isoelectric pH, proteins are positively charged and can be adsorbed by means of cation exchange mechanisms. The isoelectric pH for acid phosphatase isolated from rat liver was found to be 7.7 (Igarashi and Hollander, 1968), which is higher than the buffer pH (4) used in the assay procedure for acid phosphatase. The isoelectric pH for pyrophosphatase purified from yeast was found to be 4.75 (Butler, 1971), which is lower than the buffer pH (6) used in the assay procedure for pyrophosphatase. However, it is quite well-established that the pH in the microenvironment of an enzyme bound to a solid support can be 1-2 units lower than that in solution (McLaren and Packer, 1970). Therefore,

the pH of the microenvironment in which the acid phosphatase bound to the clay minerals performs catalysis is nearer 2-3. The pH of the microenvironment in which the pyrophosphatase bound to clay minerals performs catalysis is nearer to 4-5. These pH values are below the isoelectric pH values reported for acid phosphatase and pyrophosphatase. Thus, these enzymes would be primarily positively charged and ionic interaction with the negatively charged clay surfaces is possible. The lower K_i values observed for acid phosphatase bound to the clay minerals than for pyrophosphatase, mentioned earlier (Table 10), may be due to the acid phosphatase being more positively charged, at the buffer pH's used in this study, than is pyrophosphatase.

Data to support the hypothesis that ionic interactions occur between clay and enzymes were reported by Albert and Harter (1973). Binding isotherms for lysosyme and ovalbumin onto smectite, biotite-vermiculite, and illite compared well with sodium release curves from the same clays. The quantity of recovered sodium was proportional to the quantity of enzyme bound. Binding of protein through ionic interaction mechanisms renders the protein susceptible to strain and disruption of the three-dimensional conformation of the protein. This conformation change could cause an alteration of the catalytic site of an enzyme, decreasing its catalytic effectiveness without affecting its binding of substrate. This hypothesis is consistent with the data reported here for

inhibition of acid phosphatase and pyrophosphatase by montmorillonite and illite.

The exact nature of the ionic interaction still remains to be investigated. Several functional groups in enzyme protein are capable of ionic interaction with clay minerals. These include the amino, carboxyl, and sulfhydryl functional groups of the enzyme. Further work is needed to identify the importance of each of these functional groups on binding of enzyme to clay mineral surfaces.

SUMMARY AND CONCLUSIONS

The objectives of this study were (1) to examine the effects of metal ions on inorganic pyrophosphatase activity of soils, (2) to study the hydrolysis of organic and inorganic phosphorus compounds added to soils, (3) to study the phosphatases in plant materials, manures, sewage sludges, and soils, and (4) to study the inhibition of corn root acid phosphatase and inorganic pyrophosphatase by clay minerals.

The findings can be summarized as follows:

1. In the three soils studied in which the exchangeable and soluble cations were removed by an NH_4OAc-leaching procedure, it was found that the pyrophosphatase activity of these soils was decreased. This was thought to be mainly due to removal of some of the organic-matter bound pyrophosphatase from the soils by the NH_4OAc-leaching procedure used. The decrease in pyrophosphatase activity was from 13.8 to 4.32 for Nicollet soil, from 12.7 to 6.43 for Webster soil, and from 10.8 to 1.94 μmoles Pi released·g $soil^{-1}$·$5h^{-1}$ for Clarion soil. The effect of various metal ions and metal ion concentration on activities of pyrophosphatase in the NH_4OAc-leached soils was also studied. The results showed that the pyrophosphatase activity in the three soils studied was promoted by Ca^{2+}, Mg^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Mn^{2+}, was not affected by K^{+} and Na^{+}, and was decreased by Cu^{2+} and Fe^{2+}. With the average percentage increase in activity observed at the

optimum metal ion concentration in parentheses, the efficiency of the metal ions in promoting pyrophosphatase activity in the three soils studied in decreasing order was: Ca^{2+} (47) > Mg^{2+} (42) > Ba^{2+} (29) = Co^{2+} (29) > Ni^{2+} (27) > Zn^{2+} (20) > Mg^{2+} (16). Assay of pyrophosphatase in the NH_4OAc-leached Nicollet and Clarion soils at 50, 75, 100, and 150 m$\underline{M}$ PPi and at varying concentrations of Mg^{2+} and Ca^{2+} (0-250 m$\underline{M}$) at each PPi concentration showed that optimum activity generally occurred when the metal ion:PPi ratio was 1:1. This supports previous findings obtained for pyrophosphatase from other biological sources showing that Mg^{2+} and Ca^{2+} increase pyrophosphatase activity by activating the PPi substrate by forming a metal ion:PPi complex. This complex binds to the enzyme so that catalysis can occur. Mg^{2+} and Ca^{2+} also were found to protect pyrophosphatase in the NH_4OAc-leached Webster and Nicollet soils against heat inactivation. Addition of 0.5 ml of 200 m$\underline{M}$ $MgCl_2$ or $CaCl_2$ before heating at 90 C for times ranging from 0 to 30 min showed that less pyrophosphatase activity was destroyed than if soils were heated for this period at 90 C in the absence of Mg^{2+} and Ca^{2+}. Expressed as a percentage of the initial pyrophosphatase activity, the pyrophosphatase activity in the Webster soil heated for 30 min at 90 C in the absence of Mg^{2+} or Ca^{2+} was 42%. The corresponding activity in the presence of Mg^{2+} was 51% and in the presence of Ca^{2+} it was 57%. The pyrophosphatase activity of the Nicollet soil heated for 30 min at 90 C in

the absence of Mg^{2+} or Ca^{2+} was 30% of the initial activity. The corresponding activity in the presence of Mg^{2+} was 50% and in the presence of Ca^{2+} it was 57%.

2. The rates of hydrolysis of seven organic and two inorganic phosphorus compounds applied to soils at a rate of 500 ppm P and incubated at 20 C for various times under aerobic and waterlogged conditions were studied. Monomethyl phosphate, β-glycerophosphate, and α-D-glucose-1-phosphate were hydrolyzed at similar rates in the three soils used, but the rates were somewhat faster under aerobic than under waterlogged conditions. Organic P compounds in which two hydrogens of the orthophosphoric acid are replaced (e.g., diphenyl phosphate and bis-p-nitrophenyl phosphate) were hydrolyzed at slower rates than those in which one hydrogen is replaced (e.g., phenyl phosphate and p-nitrophenyl phosphate). The rates of hydrolysis of diphenyl phosphate were lower than that of bis-p-nitrophenyl phosphate. Of the two inorganic P compounds studied, ammonium tetrametaphosphimate did not hydrolyze in soils, and the rates of hydrolysis of phosphonitrilic hexaamide were very small (6-13% hydrolyzed in 7 days) compared with those of the organic phosphates (30-100%).

Hydrolysis of the monosubstituted P compounds added to soils was more rapid in the acid Clarion and Nicollet soils than in the calcareous Harps soil. The observed higher rates of hydrolysis of the monosubstituted P compounds in these two soils compared with that in Harps soil is due to the much higher

acid phosphatase activity in these two soils. For the di-substituted organic P compounds, the highest rates of hydrolysis were found in the Harps soil. This is due to the much greater phosphodiesterase activity in the Harps soil compared with those in the Clarion and Nicollet soils.

Several of the P compounds (monomethyl phosphate, phosphonitrilic hexaamide, and ammonium tetrametaphosphimate) contained substantial amounts of N (24.6-54.2%). Among these compounds, the rate of hydrolysis of monomethyl phosphate (cyclohexane ammonium salt) was the most rapid, with 100% of the P added to soils released after 7 days of incubation under aerobic conditions.

3. Comparisons of pH optima and kinetic parameters (K_m, V_{max}, and E_a) for acid and alkaline phosphatase, phosphodiesterase, and pyrophosphatase among plant materials, manures, sewage sludges, and soils were made. It was found that generally the optimum activity of these phosphatases in soils occurred at buffer pH values 1-2 units higher than those of the phosphatases in the organic waste materials studied. Also, results obtained from previous findings are supported by our observations that acid soils contain higher levels of acid phosphatase activity than calcareous soils, but calcareous soils contain higher levels of alkaline phosphatase activity than acid soils. The four phosphatases studied were found to obey simple Michaelis-Menten kinetics. The K_m values determined for each phosphatase in the various

sources of enzyme studied were all very similar with the exception of pyrophosphatase. The average K_m value for pyrophosphatase in soils (37.8 m$\underline{M}$) was higher than that for plant materials (10.1 m$\underline{M}$) and manures (6.3 m$\underline{M}$) (the sewage sludge samples studied did not contain pyrophosphatase activity). This higher K_m value for soils was attributed to sorption of the PPi substrate by soil constituents. The conformity of the reaction rates to simple Michaelis-Menten kinetics, however, suggest that the PPi sorption by soils is proportional to the PPi concentration.

Comparison of the average V_{max} values showed that plant materials and manures contained much greater levels of activity than soils. Based on the average V_{max} values determined, plant materials contain 580, 100, and 470 times more acid phosphatase, phosphodiesterase, and pyrophosphatase activity, respectively, than soils. On the average, manures were found to contain 10, 45, 5, and 160 times more acid and alkaline phosphatase, phosphodiesterase, and pyrophosphatase activity, respectively, than soils. However, sewage sludges contain, on the average, 4.7 times more alkaline activity and about the same level of acid phosphatase activity than soils. It was also found that soils contained very similar average V_{max} values for the four phosphatases studied, even though the amounts of the various enzymes entering the soil system may vary greatly. Plant materials did not contain detectable levels of alkaline phosphatase activity so they cannot be a

source of activity for this enzyme in soils. In addition, sewage sludges had no detectable phosphodiesterase and pyrophosphatase activity.

Comparisons of the E_a values for the four phosphatases among the plant materials, manures, sewage sludges, and soils showed that there was little variation in E_a of the four enzymes studied. With the exception of acid phosphatase in sewage sludges, the lowest average E_a was observed for alkaline phosphatase in soils (32.3 kJ/mole) and the highest for acid phosphatase in soils (42.3 kJ/mole). However, acid phosphatase in sewage sludges had an average E_a value of 50.6 kJ/mole.

The effect of corn plant material, hog manure, and sewage sludge added to Webster or Canisteo soil (3 mg/g soil) and incubated for 0 to 120 days at 25 C on acid phosphatase and pyrophosphatase activity was studied. It was found that corn plant material and hog manure significantly increased ($P < 0.05$) acid phosphatase and pyrophosphatase activity, respectively, at zero time of incubation. Acid phosphatase and pyrophosphatase activity peaked after 7-10 days of incubation as microbial growth occurred but then began to decrease. After 120 days of incubation, there was no significant difference in activity from that measured at zero time of incubation. Addition of organic waste materials to soils, therefore, seems only to affect acid phosphatase and pyrophosphatase activity for a limited time and not to cause a permanent change in the

levels of these enzymes in soils. Soils were also treated with 0.5 ml of 0.05% sodium azide along with the waste materials, and it was found that sodium azide had no effect on acid phosphatase and pyrophosphatase activity at zero time of incubation. However, activity began to decline in the azide treated samples, when compared to the other samples not treated with azide, after about 4 days of incubation and continued to decline thereafter. After 120 days of incubation, soils treated with sodium azide contained significantly ($P < 0.05$) lower levels of acid phosphatase and pyrophosphatase activity than that observed at zero time of incubation. This lower activity seems due to disruption of the microbial growth cycles that occur in soils, decreasing the amount of the phosphatases being synthesized.

4. Inhibition of acid phosphatase and inorganic pyrophosphatase obtained from sterile corn roots by clay minerals showed that the type of clay mineral had a profound influence on the mechanism of inhibition of these enzymes. The inhibition by montmorillonite and illite of acid phosphatase and pyrophosphatase was partial noncompetitive in nature. The partial noncompetitive kinetic model describes an equilibrium system where the substrate and inhibitor combine independently and reversibly to the enzyme at different sites to produce ES, EI, and ESI complexes and the ESI complex can produce product, but not as effectively as ES. At infinitely high concentrations of inhibitor and at saturating levels of sub-

strate, the K_m value obtained remains unchanged but the V_{max} value decreases by a factor β, where $\beta < 1$.

Kaolinite inhibition of acid phosphatase and pyrophosphatase was partial competitive in nature. Partial competitive inhibition occurs in a system where the substrate and inhibitor bind to the enzyme at different sites to yield ES, EI, and ESI complexes, the substrate binds to free enzyme with greater affinity than to the EI complex, and the ES and ESI complexes both yield product with equal effectiveness. At infinitely high concentrations of inhibitor and at saturating levels of substrate, the V_{max} value remains unchanged but the K_m value increases by a factor α, where $\alpha > 1$. Shaking the enzyme-clay-substrate complexes during incubation did not affect the type of kinetics observed for acid phosphatase and pyrophosphatase inhibited by the clay minerals used in this study. Shaking this mixture during incubation, however, resulted in changes of the α, β, and K_i values of these enzymes.

Inhibition of acid phosphatase and pyrophosphatase was found to be due to adsorption of these enzymes by the clay minerals. When 20 ml of universal buffer containing 20 mg of corn root per ml were allowed to react with 100 mg of clay, 97-99% of the acid phosphatase activity was found to be adsorbed by the clay minerals. When 20 ml of universal buffer containing 50 mg of corn root per ml were allowed to react

wirh 100 mg of clay, 28% (kaolinite) to 91% (montmorillonite) of the pyrophosphatase activity was found to be adsorbed by the clay minerals. This adsorption was considered ionic in nature. A study of the effect of clay extracts on acid phosphatase and pyrophosphatase activity showed that clay minerals do not release any inhibitory compound which inhibits the activity of these enzymes.

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APPENDIX

Table 24. Hydrolysis of organic and inorganic phosphorus compounds added to Clarion soil[a]

Phosphorus compound	Soil condition[b]	Orthophosphate-P formed (μg)			
		Days			
		1	2	4	7
Monomethyl phosphate	A	529	866	993	946
	WL	474	731	844	976
Phenyl phosphate	A	568	1,000	1,000	1,000
	WL	600	955	1,000	1,000
Diphenyl phosphate	A	14	45	71	114
	WL	12	35	56	76
p-nitrophenyl phosphate	A	650	1,000	1,000	1,000
	WL	354	667	865	928
bis-p-nitrophenyl phosphate	A	24	67	130	188
	WL	12	44	85	113
β-glycerophosphate	A	498	760	875	853
	WL	378	543	760	922
α-D-glucose-1-phosphate	A	407	720	900	938
	WL	408	643	814	890
Phosphonitrilic hexaamide	A	28	52	96	134
	WL	0	16	32	57
Ammonium tetrametaphosphimate	A	0	0	0	0
	WL	0	0	0	0

[a]See Part II, Description of Methods, for the experimental procedure used to obtain the data.

[b]A, phosphorus compounds incubated under aerobic soil conditions; WL, phosphorus compounds incubated under water-logged soil conditions.

Table 25. Hydrolysis of organic and inorganic phosphorus compounds added to Harps soil[a]

Phosphorus compound	Soil condition[b]	Orthophosphate-P formed (μg)			
		Days			
		1	2	4	7
Monomethyl phosphate	A	204	456	749	929
	WL	176	314	489	722
Phenyl phosphate	A	275	440	768	1,000
	WL	279	516	730	821
Diphenyl phosphate	A	53	139	238	371
	WL	25	78	114	187
p-nitrophenyl phosphate	A	653	1,000	1,000	1,000
	WL	514	725	874	892
bis-p-nitrophenyl phosphate	A	234	403	659	831
	WL	203	316	471	650
β-glycerophosphate	A	178	372	635	863
	WL	85	167	827	992
α-D-glucose-1-phosphate	A	132	304	467	736
	WL	122	181	322	510
Phosphonitrilic hexaamide	A	19	32	50	68
	WL	2	4	8	12
Ammonium tetra-metaphosphimate	A	2	8	18	29
	WL	0	2	5	11

[a]See Part II, Description of Methods, for the experimental procedure used to obtain the data.

[b]A, phosphorus compounds incubated under aerobic soil conditions; WL, phosphorus compounds incubated under water-logged soil conditions.

Table 26. Hydrolysis of organic and inorganic phosphorus compounds added to Nicollet soil[a]

Phosphorus compound	Soil condition[b]	Orthophosphate-P formed (μg)			
		Days			
		1	2	4	7
Monomethyl phosphate	A	890	956	1,000	947
	WL	689	921	980	977
Phenyl phosphate	A	725	1,000	1,000	1,000
	WL	771	954	980	1,000
Diphenyl phosphate	A	26	92	134	164
	WL	20	32	59	86
p-nitrophenyl phosphate	A	852	1,000	1,000	1,000
	WL	600	873	987	1,000
bis-p-nitrophenyl phosphate	A	141	253	400	525
	WL	101	201	300	347
β-glycerophosphate	A	794	917	940	962
	WL	535	696	860	933
α-D-glucose-1-phosphate	A	749	950	980	972
	WL	462	643	834	887
Phosphonitrilic hexaamide	A	13	35	50	96
	WL	0	5	13	32
Ammonium tetrametaphosphimate	A	2	5	6	7
	WL	0	0	0	0

[a]See Part II, Description of Methods, for the experimental procedure used to obtain the data.

[b]A, phosphorus compounds incubated under aerobic soil conditions; WL, phosphorus compounds incubated under water-logged soil conditions.

Table 27. Acid phosphatase activity in soils, plant materials, manures, and sewage sludges as affected by buffer pH[a]

Source of enzyme	Sample size (mg)	Optimum pH	Acid phosphatase activity at optimum pH[b]
Soils:			
Clarion	1,000	6.5	229
Webster	1,000	6.5	248
Okoboji	1,000	6.5	160
Canisteo	1,000	6.5	59.6
Plant materials:			
Oat	5	5.0	153
Alfalfa	5	5.0	528
Corn	5	5.0	145
Soybean	5	5.0	700
Manures:			
Cow	50	5.0	31.3
Hog	50	5.0	36.7
Chicken	50	5.0	80.5
Horse	50	5.0	300
Sewage sludges:			
Shellsburg	100	5.0	26.3
Boone	100	5.0	36.1
Des Moines	100	5.0	25.7
Ames	100	5.0	20.2

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Acid phosphatase activity is expressed as μg p-nitrophenol released · sample size indicated^{-1} · h^{-1}.

Table 28. Alkaline phosphatase activity in soils, manures, and sewage sludges as affected by buffer pH[a]

Source of enzyme	Sample size (mg)	Optimum pH	Alkaline phosphatase activity at optimum pH[b]
Soils:			
Clarion	1,000	11.0	39.7
Webster	1,000	11.0	22.2
Okoboji	1,000	11.0	197
Canisteo	1,000	11.0	140
Manures:			
Cow	10	10.0	18.0
Hog	10	9.5	13.6
Chicken	10	10.5	14.4
Horse	10	10.0	24.0
Sewage sludges:			
Shellsburg	100	11.5	183
Boone	100	11.0	36.1
Des Moines	100	11.0	86.7
Ames	100	11.0	10.2

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Alkaline phosphatase activity is expressed as μg p-nitrophenol released · sample size indicated^{-1} . h^{-1}.

Table 29. Phosphodiesterase activity in soils, plant materials, and manures as affected by buffer pH[a]

Source of enzyme	Sample size (mg)	Optimum pH	Phosphodiesterase activity at optimum pH[b]
Soils:			
Clarion	1,000	8.0	21.8
Webster	1,000	8.0	119
Okoboji	1,000	8.0	153
Canisteo	1,000	8.0	122
Plant materials:			
Oat	10	5.5	125
Alfalfa	10	5.5	192
Corn	10	5.5	130
Soybean	10	5.5	219
Manures:			
Cow	30	8.0	29.4
Hog	100	8.0	56.8
Chicken	50	7.5	29.2
Horse	50	7.5	58.1

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Phosphodiesterase activity is expressed as μg p-nitrophenol released · sample size indicated^{-1} · h^{-1}.

Table 30. Pyrophosphatase activity[a] in soils, plant materials, and manures as affected by buffer pH

Source of enzyme	Sample size (mg)	Optimum pH	Pyrophosphatase activity at optimum pH[b]
Soils:			
Clarion	1,000	7.5	295
Webster	1,000	7.5	464
Okoboji	1,000	8.0	227
Canisteo	1,000	8.0	58.1
Plant materials:			
Oat	50	5.5	1,085
Alfalfa	20	5.0	1,200
Corn	20	5.5	850
Soybean	10	5.5	860
Manures:			
Cow	75	5.5	254
Hog	20	6.0	730
Chicken	50	5.5	140
Horse	50	5.5	101

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Soil pyrophosphatase activity is expressed as μg Pi released · g soil^{-1} · 5 h^{-1} and plant and manure pyrophosphatase activity is expressed as μg Pi released · sample size indicated^{-1} · h^{-1}.

Table 31. Acid phosphatase activity in soils, plant materials, manures, and sewage sludges as affected by substrate concentration[a]

Source of enzyme	Sample size (mg)	Acid phosphatase activity at substrate concentration (mM) specified[b]				
		1.0	1.3	2.0	4.0	20.0
Soils:						
Clarion	1,000	217	256	323	435	571
Webster	1,000	220	260	303	370	455
Okoboji	1,000	143	175	220	308	443
Canisteo	1,000	45.1	55.9	72.5	105	162
Plant materials:						
Oat	4	114	141	179	247	388
Alfalfa	1	78.9	102	128	177	278
Corn	3	92.7	111	146	183	374
Soybean	1	105	130	192	249	443
Manures:						
Cow	10	6.4	8.4	10.0	14.2	28.8
Hog	10	6.0	8.8	10.0	13.2	24.4
Chicken	10	18.4	21.2	26.0	33.6	47.6
Horse	10	26.8	30.4	35.2	43.2	89.3
Sewage sludges:						
Shellsburg	100	12.8	20.4	27.6	33.4	40.0
Boone	100	14.4	15.6	18.8	25.2	45.8
Des Moines	100	11.6	13.6	15.2	27.6	42.4
Ames	100	12.6	16.4	22.0	31.2	51.2

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Acid phosphatase activity is expressed as μg p-nitrophenol released · sample size indicated^{-1} · h^{-1}.

Table 32. Alkaline phosphatase activity in soils, manures, and sewage sludges as affected by substrate concentration[a]

Source of enzyme	Sample size (mg)	Alkaline phosphatase activity at substrate concentration (mM) specified[b]				
		1.0	1.3	2.0	4.0	20.0
Soils:						
Clarion	1,000	34.0	41.7	54.1	76.9	114
Webster	1,000	20.6	26.0	35.3	54.6	97.1
Okoboji	1,000	164	200	256	370	513
Canisteo	1,000	154	182	233	303	417
Manures:						
Cow	10	57.6	66.9	76.7	108	175
Hog	10	27.2	34.4	53.7	80.5	130
Chicken	10	48.8	65.9	70.7	96.1	137
Horse	10	21.2	22.8	27.2	32.0	52.9
Sewage sludges:						
Shellsburg	100	161	176	206	220	296
Boone	100	27.0	32.4	37.8	40.2	64.8
Des Moines	100	76.2	97.8	114	153	194
Ames	100	10.0	13.6	17.6	26.8	45.8

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Alkaline phosphatase activity is expressed as μg p-nitrophenol released · sample size indicated^{-1} · h^{-1}.

Table 33. Phosphodiesterase activity in soils, plant materials, and manures as affected by substrate concentration[a]

Source of enzyme	Sample size (mg)	Phosphodiesterase activity at substrate concentration (mM) specified[b]				
		1.0	1.3	2.0	4.0	20.0
Soils:						
Clarion	1,000	9.0	10.5	12.5	15.6	20.0
Webster	1,000	63.7	74.1	88.5	109	147
Okoboji	1,000	75.2	87.7	105	132	179
Canisteo	1,000	60.2	69.4	82.0	100	127
Plant materials:						
Oat	10	36.0	46.0	54.1	62.1	76.1
Alfalfa	10	106	118	133	171	197
Corn	10	86.9	95.7	109	127	154
Soybean	10	147	158	180	215	269
Manures:						
Cow	50	20.1	24.1	29.1	35.1	47.1
Hog	50	9.0	10.4	11.2	14.8	17.3
Chicken	50	9.9	12.0	14.0	16.4	20.4
Horse	50	12.5	16.8	20.8	22.0	30.1

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Phosphodiesterase activity is expressed as μg p-nitrophenol released · sample size indicated^{-1} · h^{-1}.

Table 34. Pyrophosphatase activity in soils, plant materials, and manures as affected by substrate concentration[a]

Source of enzyme	Sample size (mg)	Pyrophosphatase activity at substrate concentration (mM) specified[b]				
		10.0	12.5	16.7	25.0	50.0
Soils:						
Clarion	1,000	115	143	172	238	333
Webster	1,000	165	227	250	333	455
Okoboji	1,000	90.9	111	147	167	250
Canisteo	1,000	28.5	34.0	41.8	52.5	66.5
Plant materials:						
Oat	50	523	582	628	652	773
Alfalfa	20	437	478	537	561	709
Corn	30	468	534	597	729	836
Soybean	10	426	492	537	667	787
		---------Substrate concentration (mM)----------				
		2.0	2.5	3.3	5.0	10.0
Manures:						
Cow	50	86.0	115	145	163	257
Hog	20	312	392	445	536	701
Chicken	50	38.0	40.8	50.8	78.5	97.0
Horse	50	51.1	68.5	75.3	84.3	125

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Soil pyrophosphatase activity is expressed as μg Pi released · g soil^{-1} · 5 h^{-1} and plant material and manure pyrophosphatase activity is expressed as μg Pi released · sample size indicated^{-1} · h^{-1}.

Table 35. Acid phosphatase activity in soils, plant materials, manures and sewage sludges as affected by temperature[a]

Source of enzyme	Sample size (mg)	Acid phosphatase activity at temperature (C) specified[b]				
		10	20	25	30	40
Soils:						
Clarion	1,000	37.0	68.5	87.0	116	205
Webster	1,000	38.7	69.3	87.4	120	215
Okoboji	1,000	26.3	48.9	63.7	86.9	154
Canisteo	1,000	6.0	10.5	13.4	18.6	32.1
Plant materials:						
Oat	5	32.4	47.2	75.1	110	156
Alfalfa	3	93.1	168	236	294	506
Corn	5	39.6	71.9	115	166	239
Soybean	2	67.3	106	153	213	262
Manures:						
Cow	50	11.6	21.3	28.8	39.6	52.9
Hog	50	9.4	15.0	17.3	24.9	38.9
Chicken	50	15.7	23.3	29.1	36.9	53.1
Horse	50	51.4	82.9	93.3	118	197
Sewage sludges:						
Shellsburg	100	5.4	9.6	11.6	16.8	25.2
Boone	100	3.2	5.6	8.8	15.6	25.6
Des Moines	100	3.2	5.8	7.6	14.4	24.0
Ames	100	1.6	4.0	6.4	8.8	17.2

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Acid phosphatase activity is expressed as μg p-nitrophenol released · sample size indicated^{-1} · h^{-1}.

Table 36. Alkaline phosphatase activity in soils, manures, and sewage sludges as affected by temperature[a]

Source of enzyme	Sample size (mg)	Alkaline phosphatase activity at temperature (C) specified[b]				
		10	20	25	30	40
Soils:						
Clarion	1,000	9.0	14.3	17.0	20.2	31.2
Webster	1,000	5.3	9.0	11.8	14.1	21.0
Okoboji	1,000	40.2	65.7	79.0	102	159
Canisteo	1,000	42.3	64.3	76.1	96.5	145
Manures:						
Cow	20	30.4	56.3	66.5	82.7	110
Hog	20	14.1	22.0	27.6	35.6	72.7
Chicken	20	30.0	50.1	61.4	78.5	104
Horse	20	11.2	16.0	19.6	26.8	59.6
Sewage sludges:						
Shellsburg	100	37.0	56.0	98.8	113	186
Boone	100	6.0	9.6	20.8	27.8	35.6
Des Moines	100	13.6	19.2	37.6	41.0	55.2
Ames	100	5.4	10.0	17.0	22.2	29.4

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Alkaline phosphatase activity is expressed as μg *p*-nitrophenol released · sample size indicated^{-1} · h^{-1}.

Table 37. Phosphodiesterase activity in soils, plant materials, and manures as affected by temperature[a]

Source of enzyme	Sample size (mg)	Phosphodiesterase activity at temperature (C) specified[b]				
		10	20	25	30	40
Soils:						
Clarion	1,000	3.3	5.8	7.8	10.3	18.3
Webster	1,000	28.9	48.2	60.7	78.7	129
Okoboji	1,000	42.8	68.3	82.7	105	162
Canisteo	1,000	26.1	45.1	55.2	71.9	113
Plant materials:						
Oat	10	20.5	40.2	48.0	66.1	88.7
Alfalfa	10	33.2	75.7	81.3	114	217
Corn	10	25.6	48.8	57.3	82.1	147
Soybean	10	37.0	89.7	110	158	208
Manures:						
Cow	50	11.0	14.4	19.4	20.9	49.3
Hog	50	5.0	7.8	9.2	10.8	37.3
Chicken	50	5.0	9.5	13.7	16.9	31.2
Horse	50	6.7	12.4	14.0	18.0	29.7

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Phosphodiesterase activity is expressed as μg p-nitrophenol released · sample size indicated^{-1} · h^{-1}.

Table 38. Pyrophosphatase activity in soils, plant materials, and manures as affected by temperature[a]

Source of enzyme	Sample size (mg)	Pyrophosphatase activity at temperature (C) specified[b]				
		10	20	25	30	40
Soils:						
Clarion	1,000	72.8	120	146	191	301
Webster	1,000	123	203	246	304	472
Okoboji	1,000	36.3	66.7	87.9	119	209
Canisteo	1,000	12.6	22.0	27.4	37.2	62.1
Plant materials:						
Oat	50	207	387	457	627	731
Alfalfa	20	165	363	415	546	826
Corn	30	195	352	435	603	826
Soybean	10	184	316	435	514	780
Manures:						
Cow	50	27.6	54.3	71.3	92.0	165
Hog	20	275	518	665	915	1,450
Chicken	50	46.0	71.8	87.3	122	198
Horse	50	38.0	60.5	90.3	106	182

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]Soil pyrophosphatase activity is expressed as μg Pi released · g soil^{-1} · 5 h^{-1} and plant material and manure pyrophosphatase activity is expressed as μg Pi released · sample size indicated^{-1} · h^{-1}.

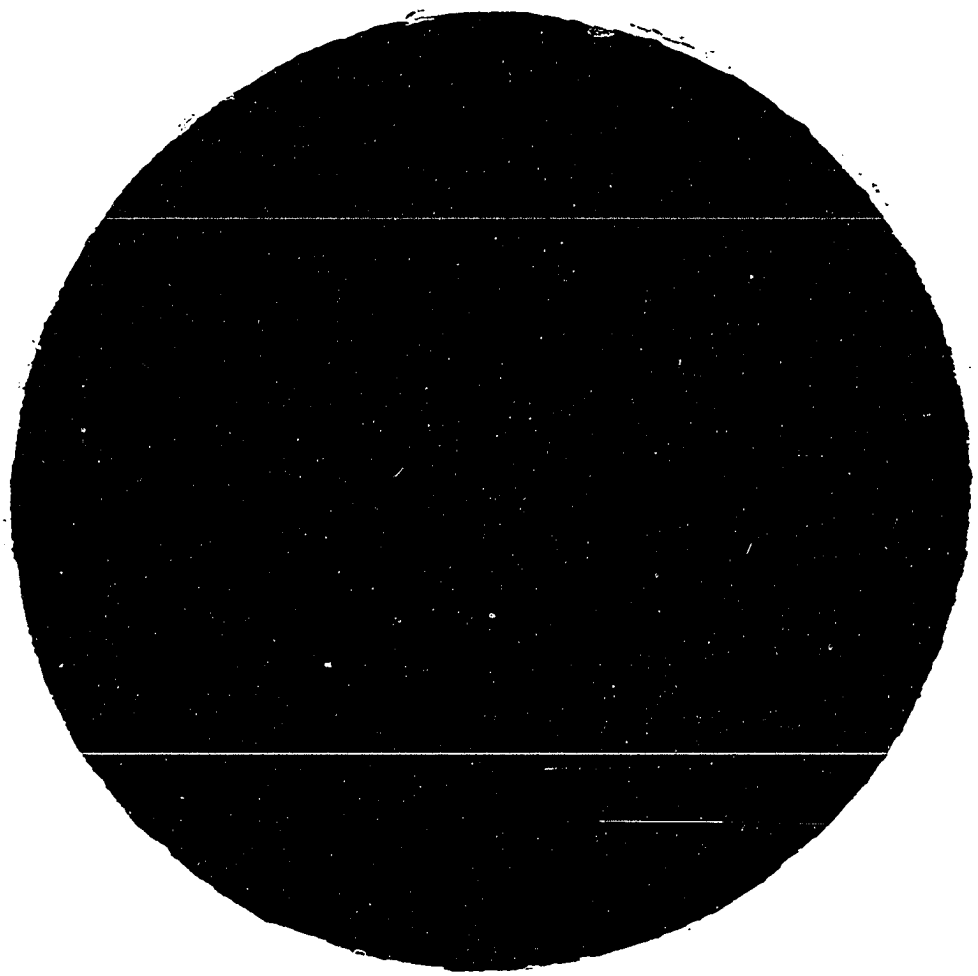

Table 39. Acid phosphatase activity as affected by various soil treatments[a]

Soil treatment[b]	Incubation time (days)											
	0	1	2	4	7	10	15	20	30	60	90	120
	Acid phosphatase activity[c]											
Canisteo soil												
None	0.50	0.52	0.41	0.42	0.42	0.60	0.61	0.46	0.44	0.40	0.38	0.35
Corn plant	0.65	0.64	0.58	0.58	0.75	0.70	0.58	0.48	0.48	0.47	0.45	0.42
Corn plant (SS)	0.50	0.47	0.54	0.55	0.70	0.70	0.58	0.49	0.49	0.47	0.45	0.42
Corn plant (N_3)	0.65	0.54	0.50	0.43	0.40	0.49	0.37	0.33	0.27	0.26	0.26	0.27
Sludge	0.46	0.47	0.53	0.51	0.44	0.73	0.65	0.57	0.55	0.45	0.45	0.42
Sludge (SS)	0.46	0.46	0.53	0.53	0.54	0.69	0.68	0.51	0.50	0.50	0.47	0.44
Sludge (N_3)	0.49	0.43	0.44	0.38	0.41	0.47	0.37	0.32	0.27	0.25	0.27	0.21
Hog manure	0.53	0.53	0.54	0.54	0.84	0.72	0.67	0.55	0.49	0.41	0.40	0.40
Hog manure (SS)	0.50	0.51	0.51	0.53	0.81	0.69	0.67	0.61	0.50	0.44	0.43	0.42
Hog manure (N_3)	0.53	0.47	0.44	0.44	0.61	0.45	0.37	0.30	0.27	0.22	0.22	0.21
Webster soil												
None	1.67	1.57	1.67	1.91	2.08	2.34	1.99	1.63	1.62	1.60	1.53	1.42
Corn plant	1.90	1.66	1.66	1.90	2.22	2.14	2.07	2.10	2.10	1.90	1.93	1.65
Corn plant (SS)	1.67	1.43	1.60	1.73	2.22	2.02	2.07	1.93	1.93	1.93	1.90	1.62
Corn plant (N_3)	1.81	1.69	1.69	1.64	1.85	1.79	1.41	1.39	1.39	1.29	1.30	1.19
Sludge	1.67	1.66	1.63	1.81	2.09	2.02	2.01	1.94	1.97	1.84	1.76	1.65
Sludge (SS)	1.67	1.60	1.64	1.67	2.21	2.02	1.98	1.98	1.87	1.79	1.76	1.70
Sludge (N_3)	1.64	1.75	1.61	1.70	1.80	1.85	1.55	1.32	1.27	1.04	1.01	0.98

Hog manure	1.73	1.73	1.55	1.69	2.26	2.11	2.06	1.98	1.89	1.75	1.73	1.36
Hog manure (SS)	1.65	1.50	1.59	1.63	2.08	2.02	2.06	1.90	1.89	1.78	1.72	1.39
Hog manure (N_3)	1.70	1.56	1.52	1.46	1.91	1.73	1.34	1.21	1.19	1.06	1.05	1.04

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]SS, soil treated with steam sterilized material; N_3, soil treated with 0.5 ml 0.05% sodium azide.

[c]Expressed as μmoles p-nitrophenol released·g soil^{-1}·h^{-1}.

Table 40. Inorganic pyrophosphatase activity as affected by various soil treatments[a]

Soil treatment[b]	Incubation time (days)											
	0	1	2	4	7	10	15	20	30	60	90	120
	Inorganic pyrophosphatase activity[c]											
Canisteo soil												
None	3.16	3.07	2.65	2.03	2.62	3.13	2.68	2.16	2.13	1.87	1.84	1.80
Corn plant	3.42	3.39	2.97	3.10	3.36	2.94	2.74	2.16	2.07	1.87	1.87	1.81
Corn plant (SS)	3.16	2.97	2.84	2.81	3.20	2.94	2.23	2.10	2.10	1.87	1.87	1.80
Corn plant (N_3)	3.43	3.00	2.68	2.45	2.07	1.55	1.49	1.52	1.59	1.45	1.29	1.28
Sludge	3.36	3.26	3.20	2.68	2.84	2.87	3.20	2.65	2.16	1.71	1.61	1.58
Sludge (SS)	3.33	3.20	2.81	2.58	3.39	2.97	1.94	1.81	1.84	1.65	1.49	1.50
Sludge (N_3)	3.36	3.00	2.20	2.32	2.39	2.20	1.71	1.55	1.45	1.39	1.39	1.29
Hog manure	4.97	4.55	2.49	2.26	2.91	2.58	2.32	1.94	1.91	1.78	1.65	1.65
Hog manure (SS)	3.26	2.91	1.94	2.29	2.78	2.62	2.26	2.20	2.16	1.87	1.65	1.60
Hog manure (N_3)	5.00	4.39	2.78	2.55	2.58	2.45	1.87	1.55	1.49	1.29	1.00	1.01
Webster soil												
None	17.8	14.7	15.8	14.8	15.5	15.3	14.6	14.5	13.9	13.8	13.9	13.5
Corn plant	18.2	16.1	13.1	13.9	18.9	17.1	16.0	15.9	16.0	15.6	15.2	15.0
Corn plant (SS)	17.6	16.0	16.1	18.5	17.2	16.7	16.2	16.1	16.1	15.6	15.0	15.0
Corn plant (N_3)	18.5	15.3	15.8	15.4	17.8	13.3	13.1	12.8	12.4	10.3	9.3	9.1
Sludge	17.7	15.3	18.8	19.0	18.2	18.1	16.4	16.1	16.5	16.2	15.9	15.3
Sludge (SS)	17.6	15.1	17.0	17.6	18.3	17.6	15.9	16.0	16.0	15.6	15.6	15.4
Sludge (N_3)	17.5	15.3	17.8	17.2	15.6	14.3	14.1	13.2	11.8	10.0	8.6	8.4

Hog manure	20.0	20.8	19.4	18.1	17.3	17.0	16.9	17.1	17.1	16.0	16.0	15.4
Hog manure (SS)	17.7	16.9	16.5	16.5	16.8	16.9	16.6	16.6	16.7	16.4	16.0	15.2
Hog manure (N_3)	20.3	20.1	18.5	19.6	16.4	15.7	14.9	14.9	14.8	12.0	10.4	9.4

[a]See Part III, Description of Methods, for the experimental procedure used to obtain the data.

[b]SS, soil treated with stem sterilized material; N_3, soil treated with 0.5 ml 0.05% sodium azide.

[c]Expressed as μmoles Pi released·g soil^{-1}·5 h^{-1}.

Table 41. Data for the inhibition of acid phosphatase by clay minerals[a]

Clay type	Assay condition[b]	Clay conc. (mg/ml)	p-nitrophenyl phosphate conc. (mM) 0.80	1.00	1.33	2.00	4.00
			--------Acid phosphatase activity[c]------				
Montmoril-lonite	NS	0	0.92	1.03	1.16	1.42	1.68
		0.2	0.54	0.58	0.72	0.82	0.98
		0.4	0.47	0.53	0.61	0.69	0.84
		1.0	0.39	0.44	0.51	0.60	0.72
		2.0	0.36	0.40	0.46	0.51	0.62
		4.0	0.30	0.33	0.37	0.42	0.53
	S	0	0.74	0.81	0.96	1.15	1.30
		0.050	0.55	0.61	0.73	0.85	1.00
		0.067	0.50	0.58	0.64	0.78	0.90
		0.10	0.45	0.51	0.57	0.69	0.84
		0.20	0.38	0.44	0.50	0.58	0.75
Illite	NS	0	0.93	1.04	1.19	1.41	1.65
		0.2	0.73	0.83	0.94	1.14	1.31
		0.4	0.65	0.73	0.82	0.98	1.16
		1.0	0.61	0.67	0.74	0.88	1.12
		2.0	0.54	0.62	0.70	0.80	0.99
		4.0	0.51	0.59	0.65	0.76	0.93
	S	0	0.92	1.01	1.14	1.24	1.66
		0.10	0.59	0.65	0.77	0.86	1.09
		0.13	0.55	0.63	0.71	0.82	1.02
		0.20	0.51	0.58	0.66	0.79	0.94
		0.40	0.44	0.50	0.56	0.69	0.79

[a]See Part IV, Description of Methods, for the experimental procedure used to obtain the data.

[b]NS, samples were not shaken during assay procedure; S, samples were shaken during assay procedure.

[c]μmoles p-nitrophenol released·20 mg homogenized corn root^{-1}·h^{-1}.

Table 41. (Continued)

Clay type	Assay condition	Clay conc. (mg/ml)	p-nitrophenyl phosphate conc. (mM) 0.80	1.00	1.33	2.00	4.00
			-------Acid phosphatase activity[c]-------				
Kaolinite	NS	0	0.97	1.09	1.26	1.53	1.79
		0.2	0.69	0.84	0.96	1.16	1.51
		0.4	0.63	0.72	0.88	1.09	1.44
		1.0	0.47	0.58	0.65	0.88	1.22
		2.0	0.39	0.47	0.56	0.77	1.16
		4.0	0.35	0.43	0.52	0.71	1.01
	S	0	1.05	1.24	1.37	1.60	2.06
		0.25	0.85	0.94	1.08	1.29	1.63
		0.33	0.81	0.88	1.01	1.17	1.54
		0.50	0.78	0.84	0.97	1.11	1.45
		1.00	0.75	0.81	0.90	1.04	1.36

Table 42. Data for the inhibition of pyrophosphatase by clay minerals[a]

Clay type	Assay condition[b]	Clay conc. (mg/ml)	Pyrophosphate concentration (mM)				
			1.33	1.67	2.23	3.33	6.67
			------Pyrophosphatase activity[c]-----				
Montmorillonite	NS	0	1.45	1.72	2.16	2.85	3.89
		1.67	0.89	1.09	1.31	1.69	2.43
		2.23	0.83	1.08	1.18	1.52	2.32
		3.33	0.76	0.92	1.12	1.42	2.06
		6.67	0.71	0.85	1.08	1.39	1.89
	S	0	2.38	2.86	3.22	3.89	5.17
		0.25	1.72	1.92	2.36	2.89	3.57
		0.33	1.61	1.81	2.16	2.70	3.33
		0.50	1.44	1.68	2.03	2.33	3.03
		1.00	1.31	1.53	1.81	2.17	2.86
Illite	NS	0	1.40	1.68	2.08	2.59	4.00
		1.67	1.32	1.46	1.75	2.23	3.33
		2.23	1.09	1.35	1.67	2.20	3.14
		3.33	0.99	1.21	1.49	1.98	2.79
		6.67	0.87	0.99	1.25	1.72	2.36
	S	0	1.98	2.30	2.80	3.37	4.42
		0.50	1.71	2.00	2.35	3.07	3.85
		0.67	1.66	1.92	2.29	2.80	3.70
		1.00	1.55	1.80	2.19	2.56	3.45
		2.00	1.32	1.55	1.83	2.32	3.11
Kaolinite	NS	0	1.38	1.62	1.89	2.50	2.95
		16.7	0.90	1.09	1.36	1.79	2.68
		22.3	0.83	1.01	1.28	1.63	2.50
		33.3	0.72	0.92	1.15	1.57	2.40
		66.7	0.67	0.85	1.07	1.47	2.08
	S	0	2.33	2.67	3.12	4.12	5.22
		8.3	1.57	1.84	2.21	2.94	4.16
		11.1	1.43	1.72	2.08	2.77	3.89
		16.7	1.30	1.56	1.91	2.56	3.70
		33.3	0.95	1.15	1.48	2.02	3.02

[a]See Part IV, Description of Methods, for the experimental procedures used to obtain the data.

[b]NS, samples were not shaken during assay procedure; S, samples were shaken during assay procedure.

[c]μmoles Pi released·50 mg homogenized corn root^{-1}·h^{-1}.